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EFFECTS OF ADRENAL CORTICAL STEROIDS ON CALCIUM METABOLISM

IRWIN CLARK,[1] RAYMOND F. GEOFFROY AND WILLIAM BOWERS

Merck Institute for Therapeutic Research, Rahway, New Jersey

ABSTRACT

Radioactive calcium (Ca^{45}) was used to investigate the effects of hydrocortisone and prednisolone on calcium metabolism in the rat. It was found that the absorption of Ca^{45} by the intestines was unimpaired by the administration of hydrocortisone. The administration of hydrocortisone or prednisolone to rats recently injected with Ca^{45} resulted in an increased urinary radiocalcium. This increase varied with the dosage, potency, and persistence of the steroid in the body. The uptake of Ca^{45} by the femurs of both normal and nephrectomized rats was inhibited by the administration of hydrocortisone. Inanition resulted in a very slight inhibition of Ca^{45} uptake by bone. Hydrocortisone exerted no observable mobilization on total bone calcium.

IT HAS been recognized for many years that the adrenal cortical hormones have an effect upon calcium metabolism. It is now generally accepted that the osteoporosis seen in Cushing's syndrome is the result of excess endogenous adrenal cortical secretion (1, 2). With the advent of the synthetic adrenal steroids, there was no reason to expect that their effects on calcium metabolism would be different from those of the hormones of endogenous origin. Indeed, hypercalciuria, spontaneous fractures and osteoporosis have been frequently observed after long-term glucocorticoid therapy (3–10). The administration of adrenal glucocorticoids to rats also results in hypercalciuria, the magnitude of which is dependent upon the dosage and potency of the steroid (11). However, fractures as a result of steroid treatment have been observed only in man. In the experiments to be reported, radioactive calcium was used in attempts to clarify the mode of action of the glucocorticoids upon calcium metabolism in the rat.

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[1] Present address: Departments of Orthopedic Surgery and Biochemistry, College of Physicians and Surgeons, Columbia University, N. Y., N. Y.

MATERIALS AND METHODS

General

Albino male rats of the Holtzman strain weighing between 160 and 250 gm. were used in all experiments. The rats were fed Purina chow or a calcium-free diet (12). In the three experiments in which urines were collected for only 24 hours, the rats were fasted but 10% glucose was added to their drinking water (distilled) as a source of calories and to insure adequate urine volumes. Analyses were carried out on individual urines although the data are expressed as the averages of the individual urines. The radioactive calcium (Ca^{45})[2] was given intraperitoneally except in the experiment where the intestinal absorption of Ca^{45} was measured. Radioactivity measurements were determined on suitable aliquots of urine and on the ashed fecal matter or bone after the ash was dissolved in 1N HNO_3. The aliquots were plated out in stainless steel planchets, air-dried (overnight) and then further dried in an oven at 80° C, for twenty-four hours. All samples were counted in a flow-gas counter for a sufficient length of time to insure a statistical accuracy of 1%. The usual corrections for background, self-absorption and radioactive decay were made.

The steroids were suspended in Merck's Aqueous Vehicle #1 and were administered orally, intraperitoneally or subcutaneously. All control rats received the same volume of Aqueous Vehicle #1 as the steroid treated animals. Whenever the steroids were administered for a week or more, subcutaneous injections of penicillin and streptomycin were given to each rat (control and treated) three times weekly to prevent infection.

Total urinary and bone calcium determinations were made with a Wechselbaum-Varney flame photometer (13).

Three types of control rats were used:

(1) *Ad libitum* feeding controls.
(2) Pair-fed controls i.e., these rats received exactly the same amount of food as did their *ad libitum* feeding mates which received hydrocortisone.
(3) Pair-weighed controls i.e., the food intakes of these rats were adjusted daily in order that their final body weights would be equal to those of the treated rats.

An analysis of variance was performed on the data of each experiment.

Specific

In the experiment in which the absorption of orally administered Ca^{45} was determined, the steroid treated rats received 2 mg. of hydrocortisone/100 gm. body weight for 7 days. During this time they were on a calcium-free diet (12). Twenty-four hours before being sacrificed, each rat received *per os* 6 μc of Ca^{45} and 1.4 mg. of calcium lactate/100 gm. body weight and urine collections were begun.

In another experiment the effect on urinary radiocalcium of a single oral dose of steroid was compared with that following a single intraperitoneal injection. Forty-eight hours prior to steroid administration each animal received 10 μc. of Ca^{45}/100 gm. Urine collections were begun immediately following treatment with the steroid.

In the experiment comparing the effects of oral and subcutaneous treatment with hydrocortisone on urinary radiocalcium, the rats received 50 μc. of Ca^{45}/100 gm. body weight subcutaneously 53 days before urine collections were begun. Five days before steroid treatment, half of the rats were placed on a calcium-free diet, and were kept on this diet throughout the experimental period of 8 days. All rats received 15 gm. of food during the period of urine collections.

In the experiment in which the effect of calcium restriction and hydrocortisone upon bone resorption was studied, the rats received 100 μc. of Ca^{45}/100 gm. body weight, 53

[2] Ca^{45} was used as the calcium chloride salt received on allocation from the Atomic Energy Commission.

TABLE 1. THE EFFECT OF PRETREATMENT WITH HYDROCORTISONE AND FOOD RESTRICTION UPON THE EXCRETION OF A SINGLE DOSE OF RADIOCALCIUM

Treatment	Hydrocortisone	Pair-weighed controls	*Ad libitum* controls
	% of injected radiocalcium excreted		
Urine	3.6**	0.59	0.66
Feces and Gastrointestinal Tract	31.5	34.5	26.9

** Significantly different from both groups of controls ($P < .01$).

days before collecting urines. Eleven days before steroid treatment, the stock diet was replaced by a calcium free diet (12). In all, the rats were maintained on this calcium-free diet for 23 days, the last twelve days being the urinary collection period. The period of pretreatment on a calcium free diet was twice as long as in the previous experiment.

In the experiment in which the effects of hydrocortisone on intact and nephrectomized rats were compared, the treated animals received 1 mg. of hydrocortisone/100 gm. body weight subcutaneously daily for three weeks. Bilateral nephrectomies were carried out via the abdominal route. Twenty-four hours before being sacirficed, each rat received 3 μc. of Ca^{45}/100 gm. body weight.

RESULTS

The effect of pretreatment with hydrocortisone on the absorption and excretion of an oral dose of radioacalcium

It can be seen from the data (Table 1) that the urinary excretion of radiocalcium by the hydrocortisone treated rats is about 5 times greater than that of either group of control rats ($P < .01$). There was no significant difference in combined intestinal content and fecal excretion of radiocalcium between the three groups.

Comparison between the effects of a single oral and intraperitoneal injection of hydrocortisone and prednisolone on urinary radiocalcium forty-eight hours after the administration of Ca^{45}

As can be seen from Table 2, the administration of both steroids resulted in an increase in radiocalcium excretion above the control values during the first 8 hours, especially after oral administration as compared to intra-

TABLE 2. THE EFFECTS OF AN ORAL AND INTRAPERITONEAL INJECTION OF HYDROCORTISONE AND PREDNISOLONE ON URINARY RADIOCALCIUM

Treatment	No. of animals	I.P.	Oral	I.P.	Oral
		Hours			
		0–8	0–8	8–24	8–24
Controls*	6	100	100	100	100
Hydrocortisone—2 mg./100 gm.	6	137	253**	89	95
Prednisolone—0.5 mg./100 gm.	6	136	230**	91	103

Radiocalcium was given 48 hours before urine collections were begun.
* The control count values were arbitrarily set at 100 for ease in comparison of the data.
** Significantly different from the control values ($P < .01$).

peritoneal injection. However, the excretion of Ca^{45} was comparable to the controls during the 8- to 24-hour period. Although prednisolone was given in 1/4 the dosage of hydrocortisone, it evoked about the same response.

The effect of hydrocortisone on urinary Ca^{45} and Ca^{40} of rats which had received Ca^{45} fifty-three days before steroid treatment

Singer and Armstrong have shown that about 53 days after the administration of radiocalcium to rats, the urinary excretion of the isotope represents a small uniform fraction of the administered dose (14). Any increase in urinary radiocalcium above the control values must be derived

TABLE 3. THE EFFECT OF HYDROCORTISONE ON THE URINARY EXCRETION OF Ca^{45} AND C^{40} OF RATS KEPT ON STOCK OR CALCIUM-FREE DIET, FIFTY-THREE DAYS AFTER THE ADMINISTRATION OF THE ISOTOPE

Weight change	Stock diet				Calcium-free diet		
	Control −20 gm.	Hydrocortisone (s.c.) −79 gm.		Hydrocortisone (oral) −31 gm.	Control −48 gm.	Hydrocortisone (s.c.) −99 gm.		Hydrocortisone (oral) −79 gm.
No. of Rats	4	4		4	4	4		4
		Total counts excreted				*Total counts excreted*		
Urinary Ca^{45}			*P values*				*P values*	
1 & 2 days	37,000	18,000	<.05	42,000	24,000	26,000	N.S.	27,000
3 & 4 days	59,000	24,000	<.01	44,000	33,000	24,000	N.S.	25,000
5 & 6 days	79,000	92,000	N.S.	73,000	43,000	134,000	<.05	36,000
7 & 8 days	52,000	75,000	N.S.	46,000	38,000	145,000	<.01	34,000
Sum 0–8	227,000	209,000		205,000	138,000	329,000	<.05	122,000
Urinary Ca^{40}	*mg.*	*mg.*	*P values*	*mg.*	*mg.*	*mg.*	*P values*	*mg.*
1 & 2 days	4.6	5.5	N.S.	5.1	3.0	3.9	N.S.	3.4
3 & 4 days	5.0	6.1	N.S.	5.5	3.1	3.6	N.S.	3.2
5 & 6 days	7.2	10.4	<.05	6.3	4.4	6.3	<.05	4.9
7 & 8 days	4.9	8.9	<.05	4.9	3.8	6.5	<.01	4.2
Sum 0–8	21.7	30.9	<.01	21.8	14.3	20.3	<.05	15.2

The rats received Ca^{45} 53 days before treatment was begun.

from the labeled calcium in bone, since this site is the body's principal reservoir of calcium.

After subcutaneous administration of hydrocortisone (Table 3), the usual delay or depression of urinary radiocalcium was observed for the first four days (11). This depression of urinary radiocalcium was significant in the rats maintained on a stock diet ($P < .05$ and $P < .01$) whereas, in the rats maintained on a calcium-free diet, no differences were observed. Beginning at day 5, and continuing through day 8, a significant increase in total calcium^{40} was observed in all rats which received the steroid subcutaneously. A statistically significant increase in urinary Ca^{45} was observed only in those rats which were kept on a calcium-free diet. Those on stock diet tended to have increased urinary Ca^{45} but the results were not significant.

The oral administration of hydrocortisone to rats on either stock or calcium-free diet, had no significant effect upon Ca^{45} or total calcium excretion.

The greater effectiveness of the subcutaneous route as compared to the

oral is also evident by the greater weight loss, despite the fact that all animals consumed the same amount of food.

The effect of calcium restriction and hydrocortisone upon bone Ca^{45}

Here too, the isotope was administered 53 days before the steroid treatment was inaugurated. Thus, any increase in urinary radiocalcium again must be derived from bone.

There was no increase in urinary radiocalcium until day 5. From day 5 through day 12, there was a significant increase in urinary radiocalcium ($P < .01$) (Table 4).

TABLE 4. THE EFFECT OF HYDROCORTISONE UPON URINARY EXCRETION OF Ca^{40} AND Ca^{45} OF RATS KEPT ON A CALCIUM-FREE DIET

Body weight change, Gm.	Controls +40	Hydrocortisone 2 mg./100 gm. −50	P Values
	Counts excreted		
Urinary Ca^{45}			
1 through 4 days	35,000	34,000	N.S.
5 through 8 days	27,000	47,000	<.01
9 through 12 days	27,000	63,000	<.01
Sum 0–12	89,000	144,000	<.01
Urinary Ca^{40}, mg.			
1 through 4 days	5.5	4.7	
5 through 8 days	5.0	5.0	
9 through 12 days	5.1	5.4	
Sum 0–12	15.6	15.1	

The rats received Ca^{45} 53 days before urine collections.

In this experiment the rats received the calcium-free diet for 11 days rather than 5 days before urine collections were started. As a result of this longer pretreatment with a calcium-free diet, there was no difference in the total calcium excretion between the steroid treated rats and their controls despite the disparity in weight change.

Comparative effects of food restriction and hydrocortisone treatment upon the uptake and excretion of radiocalcium in normal and nephrectomized rats

The serum radioactivities, urinary radioactivities, and femur specific activities of the hydrocortisone treated intact rats were significantly different ($P < .01$) from those of either group of controls (Table 5). There was no difference between the pair-weighed controls and *ad libitum* feeding controls in the excretion of radioactive calcium, despite a difference in weight gain of 57 gm.

There were significant differences in serum radioactivities and femur specific activities between the nephrectomized *ad libitum* feeding controls and the nephrectomized hydrocortisone treated rats. These differences

were of the same order as those seen in the intact rats. The serum activities of the nephrectomized pair-weighed controls were no different from those of the nephrectomized steroid treated rats. However, there were differences in the specific activities of the femurs ($P < .05$).

DISCUSSION

If hydrocortisone interferes with the intestinal absorption of calcium in the rat, one would expect to find after a single oral dose of Ca^{45}, more Ca^{45} in the gastrointestinal tract and feces and less in the urines of the steroid treated rats than in the control animals. However, this is not the case (Table 1) where about 5 times more isotope is found in the urines of the

TABLE 5. THE EFFECT OF HYDROCORTISONE ON THE UPTAKE OF RADIOCALCIUM BY THE FEMURS OF INTACT AND NEPHRECTOMIZED RATS

Treatment	No. of rats	Body wt. change, gm.	Serum activity, c./min./ml.	Urine activity, c/24 hr. sample	Bone specific c./min./mg. Ca
Intact animals					
Hydrocortisone 1 mg./100 gm.	8	+ 24	7860	1.53×10^5	1160
Pair-weighed controls	8	+ 30	5630**	0.51×10^{5}**	1560**
Ad libitum feeding controls	8	+ 87**	6105**	0.47×10^{5}**	1720**
Nephrectomized animals					
Hydrocortisone 1 mg./100 gm.	8	+ 31	7295	—	1483
Pair-weighed controls	8	+ 30	7085	—	1843*
Ad libitum feeding controls	8	+110**	5590**	—	2110**

The rats received Ca^{45} twenty-four hours before being sacrificed.
* Significantly different from hydrocortisone treatment ($P < .05$).
** Significantly different from hydrocortisone treatment ($P < .01$).

hydrocortisone treated rats, and no increase is seen in the gastrointestinal tract and feces. It appears that adequate absorption of calcium takes place but that there is an inhibition of the utilization of Ca^{45} which is reflected by the greater amount of isotope appearing in the 24-hour urines of the treated rats.

It has been reported that the administration of the adrenal cortical steroids to rats invariably caused an increase in urinary radiocalcium, when the isotope was administered shortly before the steroid (11). Forty-eight hours after the injection of Ca^{45}, the level of circulating isotope was high and the isotope was still being incorporated into bone (14). If it is prevented from doing so, it will be excreted and presumably this is what occurred here (Table 2). Further evidence for the decreased utilization of Ca^{45} under the influence of hydrocortisone may be found in the experiment using nephrectomized rats (Table 5). Although the serum radioactivity levels are significantly higher in the hydrocortisone treated rats, (both intact and nephrectomized) the specific activities of the bones are signifi-

cantly less, indicating less incorporation of isotope. Moreover, the steroid treated rats excreted much more Ca^{45} in their urine. In the intact rat, the possibility does exist that the decreased uptake of Ca^{45} by bone may be the result of the increased urinary Ca^{45}, however, this is not possible in the rats which had their kidneys removed.

Prednisolone, although administered at one quarter the dose level of hydrocortisone, has an equal effect upon urinary radiocalcium (Table 2). Clinically, prednisolone is about 4 times more active than hydrocortisone (15). Presumably, the more potent is the steroid in inhibiting bone formation, the greater will be its influence in preventing calcium utilization.

It has been shown that the duration of adrenal cortical steroid action depends on the route of administration (18). After daily subcutaneous injections, there was a slow continued release of the relatively insoluble steroids enabling them to continue their metabolic effects throughout the experimental period. However, after oral or intraperitoneal administration, there is a rapid disappearance of the steroid and its effects. Thus, the increased urinary Ca^{45} observed after oral or intraperitoneal injections at 8 hours is no longer evident at the end of 24 hours (Table 2). Even after 8 days of oral administration of hydrocortisone, there is no observable effect on radiocalcium excretion, indicating the rapidity with which the activity of the steroid disappears in the rat (Table 3). However, a significant increase is seen after subcutaneous administration (Table 3). This increase in urinary radiocalcium can only come from the bone since the animals received the isotope 53 days before. Although there appears to be an increase in Ca^{40} excretion on a calcium-free diet after subcutaneous administration of the steroid (Table 3), this does not occur if the animals are kept for a longer period of pretreatment on a calcium-free diet (Table 4). In this experiment, the rats were kept on a calcium-free diet for 11 days before being treated. Even after 12 days treatment with hydrocortisone at a level of 2 mg./100 gm. body weight subcutaneously, there is no increase in Ca^{40} excretion. Irving (19), has pointed out the importance of careful dietary preconditioning before patients can be used for calcium excretion studies. However, there is a striking increase in Ca^{45} excretion beginning at day 5 and continuing through day 8. This 4-day delay in the increased urinary Ca^{45} may be explained by assuming this is the time necessary for the normal resorptive processes in the bones to reach the site of the deeper areas of calcification which were formed 53 days earlier (20). The increased urinary radiocalcum must be derived from the Ca^{45} liberated by the normal resorptive or remodelling processes. However, under the influence of the steroid it is not re-utilized as efficiently and hence is excreted in the urine. If bone destruction had occurred, one would expect an increase in the total Ca^{40} in the urines of the treated animals. Since there is no detectable increase in total urinary calcium over that of the controls, the possibility that hydrocortisone mobilizes calcium from bone seems unlikely.

Assuming the calcium content of the skeleton to be 1% of the body

weight, a 300 gm. rat would contain about 3 gm. of calcium. Taking the usual figure of about 30% skeletal calcium loss before osteoporosis can be recognized in man, a loss of 900 mg. would be required before osteoporosis would become manifest in the rat. Since the hydrocortisone treated rats lose only about 10 mg. Ca^{40} more than the controls in 8 days (Table 3), and approximately the same amount more via the feces, (unpublished) this would require about 360 days for these rats treated with 2 mg. of hydrocortisone daily to lose 900 mg. of calcium. It is possible that this may be part of the reason why the typical pathologic picture of osteoporosis has not been observed in the rat (16, 21) after steroid treatment, since to the best of our knowledge, rats have not been kept on the adrenal steroids for this length of time.

The exact mechanism whereby the adrenal cortical hormones inhibit calcium from being used for new bone synthesis is at present unknown. Albright (2) has hypothesized that the primary effect of adrenal cortical action on bone is to inhibit the synthesis of bone protein. It has been shown that the steroids do interfere with the synthesis of new protein (17). Perhaps the limiting factor may be in the synthesis of chondroitin sulfate which is not made as readily when steroids are administered (22). The primary site of adrenal cortical steroid action on bone still remains to be elucidated.

REFERENCES

1. Albright, F., W. Parson and E. Bloomberg: *J. Clin. Endocrinol.* **1**: 375. 1941.
2. Albright, F.: *Harvey Lect.* **38**: 123. 1942–3.
3. Irwin, J. W., P. H. Henneman, D. M. K. Wang and J. Burrage: *J. Allergy* **25**: 201. 1954.
4. Boland, E. W. and N. E. Headly: *J.A.M.A.* **144**: 365. 1950.
5. Soffer, L. J. and R. Bader: *J.A.M.A.* **149**: 1002, 1952.
6. West, H. F. and G. R. Nevins: *Lancet* **265**: 1123. 1953.
7. Bunim, J. J., M. Zoff and C. McEwen: *Am. J. Med.* **18**: 27. 1955.
8. Demartini, F., A. W. Grokoest and C. Ragan: *J.A.M.A.* **149**: 750. 1952.
9. Teicher, R. and C. T. Nelson: *J. Invest. Dermat.* **19**: 205. 1952.
10. Albright, F. and P. H. Henneman: *Trans. Macy Conference on Metabolic Interrelations* **5**: 191. 1953.
11. Clark, I. and R. F. Geoffroy: *Fed. Proc.* **16**: 548. 1957.
12. Munson, P. L., O. A. Iseri, L. W. Parker and R. O. Greep: *J. Dent. Research* **31**: 463. 1952.
13. Chen, P. S., Jr. and T. Y. Toribora: *Analyt. Chem.* **25**: 1642–1644. 1953.
14. Singer, L. and W. D. Armstrong: *Metabolic Interrelations, Josiah Macy Foundation*, p. 259, 1951.
15. Bunim, J. J., M. M. Pechet and A. J. Bollet: *J.A.M.A.* **157**: 311–318. 1955.
16. Winter, C. A., R. H. Silber and H. C. Stoerk: *Endocrinology* **47**: 60. 1950.
17. Clark, I.: *J. Biol. Chem.* **200**: 69. 1953.
18. Porter, C. C. and R. H. Silber: *Endocrinology* **5**: 373. 1953.
19. Irving, J. T.: *Calcium Metabolism, Methuen Monograph*, 1957.
20. Clark, I. and R. F. Geoffroy: *J. Biol. Chem.* **233**: 203. 1958.
21. Follis, R. H., Jr.: *Proc. Soc. Exp. Biol. and Med.* **76**: 722. 1951.
22. Layton, L.: *Proc. Soc. Eper. Biol. and Med.* **76**: 596. 1951.

QUANTITATIVE *IN VITRO* RESPONSE OF THYROID TISSUE TO THYROTROPIC HORMONE[1]

KATHLEEN T. HART, DEA DRUET AND ROBERT E. MACK

Radioisotope Laboratory, St. Louis V. A. Hospital, and the Department of Medicine, St. Louis University, School of Medicine, St. Louis, Missouri

ABSTRACT

In vitro uptake of iodine by beef thyroid minces was measured and found to be proportional to thyrotropic hormone content of the incubation medium. Such a response to TSH was not elicited from thyroid tissue in the form of homogenates. After two hours incubation in 4.0 ml. of Krebs-Ringer-phosphate buffer containing 10 to 20 μc. NaI^{131}, 800 mg. of minced beef thyroid displayed a mean iodine uptake of 22.6% (12.0 to 35.9) in the absence of added thyrotropic hormone. In medium containing 10 m.u./ml. of thyrotropin, mean uptake was 40.2% (22.0 to 69.3). When thyrotropic hormone concentration expressed in logarithms was plotted against % uptake there was a rectilinear response between 1 and approximately 10 m.u./ml. of TSH. The regression coefficient, determined by the responsiveness of the gland, was different for each mince. Analysis of variance of the regression lines indicated a very significant relation between dose and response. In this incubation medium iodine uptake was depressed by 6×10^{-4} M KI. Thyroxine at 4×10^{-5} M influenced neither initial iodine uptake nor response to thyrotropic hormone. Uniformity of response of thyroid minces and the magnitude of alterations in iodine uptake induced by TSH suggest the applicability of these techniques for *in vitro* estimation of thyrotropic hormone.

THYROID tissue has the unique ability to concentrate iodide converting it into iodine-containing organic compounds. The chemical reactions associated with such changes are dependent on several factors. Some are humoral and others are confined to the thyroid itself. A normally functioning gland is stimulated by thyrotropic hormone to take up iodide and to discharge the iodine-containing hormones into circulation. Chemical changes taking place during synthesis of these hormones have been investigated with I^{131} labeled iodide under both *in vivo* and *in vitro* conditions. A comparison of the compounds formed indicates that the reactions occurring in thyroid tissue are determined by viability, and in the case of thyroids studied under *in vitro* conditions by the degree of subdivision of tissue.

Although there is a difference in the compounds formed, it has been found that under both *in vivo* and *in vitro* conditions thyrotropic hormone

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increases utilization of I^{131}. Botkin *et al.* (1) examined thyroid glands removed from rats after administration of NaI^{131} and reported that the radioiodine content was related to previous exposure to thyrotropic hormone. Roche *et al.* (2) demonstrated that thyroid glands of rats cultured *in vitro* were stimulated by the anterior pituitary to synthesize iodothyronines. Effects of thyrotropic hormone on collection and release of iodide from beef thyroid glands were studied by Bakke and Lawrence (3). Bottari (4) reported that portions of thyroid incubated *in vitro* with thyrotropic hormone exhibited a degree of utilization of I^{131} proportional to the concentration of tropic hormone.

A single gland is not necessarily homogenous with respect to distribution of iodine-concentrating ability. A non-uniform binding of iodine in the whole gland has been indicated by Joftes and Warren (5) who found that in hamster thyroid more radioiodine was concentrated in the central follicles than those on the periphery. For accurate *in vitro* measurement of the response of thyroid tissue to thyrotropic hormone it was desirable to reduce tissue variability to a minimum. Therefore, in the study presented here several glands were employed and they were minced together to further reduce variability. The minces were incubated with I^{131} in media supplemented with various amounts of thyrotropic hormone and uptake of radioiodine was measured.

METHODS

Beef thyroid glands removed from animals within 30 minutes after death were brought to the laboratory in ice cold buffer. The buffer used in all experiments was Krebs-Ringer-phosphate pH 7.4 in which the .11 M $CaCl_2$ solution was replaced by an equal volume of 0.9% NaCl. For all manipulations ice cold solutions were employed. After the glands were freed of extraneous tissue they were minced with scissors and suspended in buffer. Following this washing, 800 mg. portions of the minced thyroid were weighed into 25 ml. Erlenmeyer flasks. To these were added buffer containing thyrotropic hormone at the level of 0 to 100 m.u.[2]/ml., 10 to 20 μc. of NaI^{131} and any other reagent which was to be examined for its influence on I^{131} uptake. The final volume was adjusted to 4.0 ml. with buffer. These flasks were incubated in the Dubnoff shaker at 37° C for two hours in an atmosphere of 95% O_2–5% CO_2. At the end of this period the tissue was immediately processed for measurement of radioactivity. The incubation mixture was transferred to a test tube with care to remove all tissue. The suspension was centrifuged for 20 min. at 2950 r.p.m. and the supernatant decanted. Tissue was resuspended in 5 ml. of buffer and centrifuged as previously. The washing was repeated twice. These manipulations were conducted at room temperature and required $2\frac{1}{2}$ to 3 hours. Radioactivity was determined utilizing a probe type scintillation counter at a fixed distance from tissue. A solution of I^{131} equal to that added to the incubation medium was also counted and from these values the % uptake of iodine was calculated.

RESULTS

Increased amounts of radioactivity were taken up by thyroid minces fol-

[2] m.u.—An abbreviation for milliunit, one-thousandth of a U.S.P. unit of commercially available thyrotropic hormone.

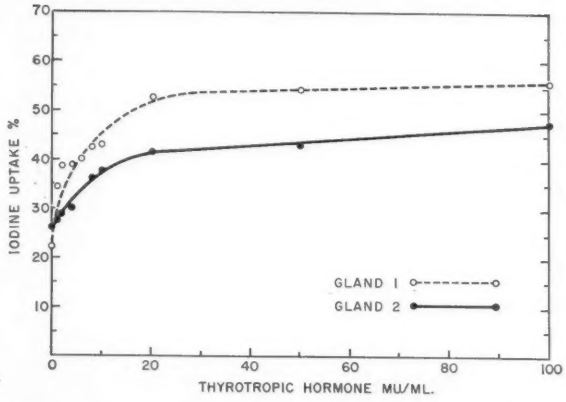

FIG. 1. *In vitro* I^{131} uptake of beef thyroid minces incubated in 0–100 m.u./ml. of thyrotropic hormone (TSH expressed as m.u./ml.)

lowing their incubation with TSH and I^{131}. The degree of uptake elevation was determined by the thyrotropic hormone content of the medium. Responses of typical thyroid minces to TSH at levels of 0 to 100 m.u./ml. are illustrated in Figure 1. Here it can be seen that iodine uptake increased in the presence of the tropic hormone until tissue radioactivity approached a maximum. This relation between dose and response may be shown as a sigmoid curve if % uptake is plotted against TSH expressed in logarithms (Fig. 2). From inspection of each curve the linear portion can be determined. In over 80% of a group of 60 minces response was linear from 1 to 8 or 10 m.u./ml. of TSH (Fig. 3). These are typical lines and show the effect on different thyroid minces of increasing concentrations of TSH. Regression coefficients of the minces which were studied ranged from 5.6 to 33.7. Application of the *t* test to iodine uptake at 0 and 1 m.u./ml. indi-

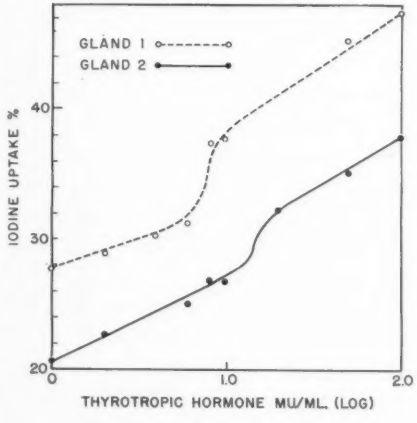

FIG. 2. *In vitro* I^{131} uptake of beef thyroid minces incubated in 0–100 m.u./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

cated that it was possible to detect thyrotropic hormone at the level of 1 m.u./ml. ($P < .001$ to .005) in the incubation medium.

Average uptakes of radioiodine of a group of 60 minces are summarized in Table 1. Iodine uptake rose from $22.6 \pm 5.3\%$ in the absence of added hormone to $47.4 \pm 7.8\%$ when the medium contained 100 m.u./ml. of tropic hormone. Composite uptake data are plotted in Figure 4. There was

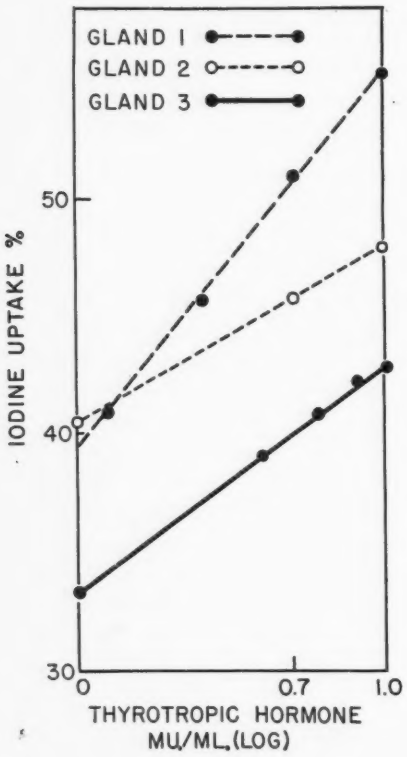

FIG. 3. *In vitro* I^{131} uptake of three different thyroid minces incubated in 0–10 m.u./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

a linear relation of dose and response between 1 and 8 m.u./ml. of thyrotropic hormone. An analysis of variance of the line indicated a P of .008 for the line, a figure which resulted from the variations in different glands. Such tissue differences were lacking when a single mince was employed. Homogeneity of a mince was reflected in a comparison of standard deviations in uptake of many minces with the deviation of duplicate samples of the same mince. Two to six samples of a single mince containing equal concentrations of tropic hormone were employed over the range 0 to 100 m.u./ml. and the standard deviation in uptake at every level of TSH for each mince was calculated. The average deviation of 20 such sets of figures at each tropic hormone level is found in Table 2. Also included are the

TABLE 1. EFFECT OF THYROTROPIC HORMONE ON IODINE UPTAKE OF THYROID MINCES

TSH conc. m.u./ml.	% uptake I^{131}	
	Range	Mean ± std. dev.
0	12.0–35.9	22.6 ± 5.33
1	11.7–33.4	24.6 ± 5.18
2	14.8–39.3	27.3 ± 6.30
4	19.6–49.9	31.1 ± 7.99
6	20.7–56.7	35.3 ± 9.00
8	22.6–52.4	37.1 ± 8.38
10	22.0–69.3	40.2 ± 12.09
20	25.8–69.0	43.3 ± 11.82
50	33.9–68.0	46.0 ± 8.81
100	38.0–67.2	47.4 ± 7.82

standard deviations which resulted when uptake of 60 different minces were compared. The latter figures are five to six times as great as deviations of individual minces. Uniformity in uptake of duplicate samples gave indication of homogeneity of a mince. Because a single mince gave uniform response and each mince differed from the next with respect to both initial iodine uptake and response to thyrotropic hormone, a separate regression line was applied to each mince. An analysis of variance of such lines has indicated a $P < .001$ for the regression line, a figure which indicates a significant dose-response relation.

To account for the entry of I^{131} measured ultimately in thyroid minces, determination was made of the total uptake as opposed to the I^{131} which entered tissue during the washing period. For this study thyroid minces were incubated in the presence of different concentrations of thyrotropic hormone but without I^{131}. The latter was added at the end of the incubation period and the tissue washed by the usual procedure. Aliquots of incubation fluid and the three wash solutions as well as the tissue itself were examined for radioactivity. As seen in Table 3, four such studies indicated

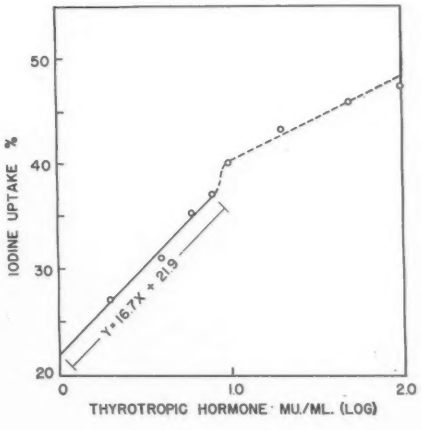

FIG. 4. Composite line representing *in vitro* iodine uptake of 60 different beef thyroid minces incubated in 0–100 mU./ml. of thyrotropic hormone (TSH expressed as logarithm of m.u./ml.)

TABLE 2. DEVIATIONS IN IODINE UPTAKE OF THYROID MINCES

TSH conc. m.u./ml.	% standard deviation	
	Pooled minces	Av. of 60 different minces
0	0.85	5.33
1	1.07	5.18
2	1.21	6.30
4	1.53	7.99
6	1.80	9.00
8	1.96	8.38
10	1.94	12.09
20	2.02	11.82
50	1.62	8.81
100	1.32	7.82

that with increasing concentrations of TSH slightly greater amounts of radioactivity entered tissue. Iodine taken up may be bound to protein or remain in the form of iodide. The trichloroacetic acid precipitable I^{131} in tissue and the combined incubation fluid and wash solutions was determined. Each 800 mg. of tissue was homogenized in buffer in an ice cooled Waring blendor. The suspension, 20 ml., was treated with 6.0 ml. of 40% trichloroacetic acid and the precipitate washed twice with 8.0 ml. of 5% trichloroacetic acid. The incubation fluid combined with solutions removed after washing the tissue (total volume approximately 20 ml.) was treated with trichloroacetic acid in the same manner. Radioactivity of the precipitates and aliquots of supernates was measured. As seen on Table 4 approximately 3% of the radioiodide added at the beginning of the washing period was precipitated with trichloroacetic acid. This value represented protein bound iodine, as well as adsorbed iodide and indicated that condi-

TABLE 3. DISTRIBUTION OF I^{131} ADDED TO THYROID TISSUE *in vitro*

Conc. TSH m.u./ml.	Fraction	Time of I^{131} addition	
		At beginning of 2 hr. incubation	At end of incubation
0	Tissue	26.8±2.3	8.7±1.4
	Supernatant	35.7±1.7	64.6±1.7
	1st Wash	14.5±2.2	13.7±3.8
	2nd Wash	11.6±0.3	7.1±0.1
	3rd Wash	8.9±3.1	4.7±1.0
10	Tissue	60.3±1.6	13.5±3.6
	Supernatant	16.3±0.8	61.8±4.8
	1st Wash	7.5±1.7	9.3±1.6
	2nd Wash	7.1±0.3	7.3±0.1
	3rd Wash	6.9±1.2	5.5±1.6
20	Tissue	69.0±1.7	14.9±2.5
	Supernatant	12.9±1.3	60.5±3.1
	1st Wash	5.0±1.3	9.1±1.3
	2nd Wash	5.4±0.4	7.3±0.4
	3rd Wash	5.8±1.5	5.7±1.5

These figures represent % of added radioiodide and are the average of four experiments.

tions which existed during the washing period were not conducive to conversions of large quantities of iodide to organic molecules.

The nature of radioiodine in tissue after incubation under the usual conditions i.e. in the presence of NaI^{131} and different concentrations of thyrotropin has been determined by applying similar techniques of trichloroacetic acid precipitation to the tissue as well as to the combined incubation and wash solutions. Data in Table 4 indicate that during incubation radioiodine precipitated with trichloroacetic acid was increased in the presence

TABLE 4. NATURE OF IODINE COMPOUNDS PRODUCED AFTER *in vitro* INCUBATION OF THYROID TISSUE

Time of addition of I^{131}	Conc. TSH m.u./ml.	Fraction	Behavior in TCA	
			Precipitated	Soluble
Beginning of Incubation	0	Tissue	13.8	13.3
		Supernate	2.6	67.0
		Total	16.4	80.3
	10	Tissue	48.3	13.1
		Supernate	3.8	33.2
		Total	52.1	46.3
	20	Tissue	57.8	12.6
		Supernate	4.5	22.6
		Total	62.3	35.2
End of Incubation	0	Tissue	1.7	6.8
		Supernate	0.5	88.1
		Total	2.2	94.9
	10	Tissue	2.5	10.9
		Supernate	0.5	78.2
		Total	3.0	89.1
	20	Tissue	2.4	12.1
		Supernate	0.6	79.3
		Total	3.0	91.4

Figures represent % added radioiodide and are the average of 4 experiments.

of thyrotropic hormone. If one compares the trichloroacetic acid precipitable I^{131} in tissue (Table 4) with the total radioiodine content (Table 3), approximately 50% (13.8/26.8) was protein bound in the absence of added thyrotropin. Increase of this fraction to 80% (48.3/60.3) was found when the preparation was supplemented with 10 m.u./ml. of TSH and to 84% (57.8/69.0) when 20 m.u./ml. were added. These figures agree with the well established relation of thyrotropic hormone to iodine uptake and utilization.

The *in vitro* iodine uptake of an isolated thyroid gland was determined in part by the degree of subdivision of tissue. Uptakes of iodine by slices, minces and homogenates were compared. In this experiment a lobe of the thyroid was divided into equal parts. Frozen and fresh slices were prepared, the former with a freezing microtome set to give slices of 250 μ. Fresh slices 0.5 mm. thick were prepared using the Stadie-Riggs microtome.

TABLE 5. COMPARISON OF IODINE UPTAKE BY THYROID MINCES, SLICES AND HOMOGENATES

Samples/ Datum	TSH m.u./ml.	% uptake ± standard deviation			
		Form of tissue			
		Mince	Frozen slice	Fresh slice	Homogenate
5	0	18.4 ± 1.0	8.3 ± 2.4		
	10	35.2 ± 6.6	8.9 ± 1.3		
4	0	18.2 ± 1.1		13.9 ± 1.0	4.23 ± 0.9
	10	32.7 ± 2.5		32.2 ± 6.6	4.40 ± 0.6
5	0	30.6 ± 2.5	12.1 ± 0.7	21.4 ± 5.2	5.90 ± 0.2
	10	49.2 ± 2.7	12.1 ± 0.6	44.7 ± 10.3	5.97 ± 0.5

Eight hundred mg. of slices were incubated in each flask. Another portion of the gland was homogenized using 400 mg. of tissue per ml. of buffer. This was accomplished using an ice-cooled Waring blendor operated for 5 minutes. For incubation, 2 ml. aliquots were employed. As control, an equal portion of the same gland was minced by the usual technique. Radioactive iodine as well as thyrotropic hormone at levels of 0 and 10 m.u./ml. were added and the volume adjusted to 4.0 ml. with buffer. After incubation the homogenate was precipitated with 1.5 ml. of 40% trichloroacetic acid and the precipitate washed twice with 5 ml. of 5% trichloroacetic acid. These data (Table 5) indicate that I^{131} was taken up by thyroid tissue in all forms and that thyrotropic hormone increased uptake in fresh slices and minces but not frozen slices or homogenates. When tissue was in the latter forms the quantity of I^{131} concentrated was less than in fresh slices or minces.

TABLE 6. EFFECT OF VARIOUS REAGENTS ON *in vitro* I^{131} UPTAKE BY THYROID MINCES

Reagent Tested	TSH conc. m.u./ml.		
	0 % response*	10 % response	20 % response
5×10^{-4} M FMN	108.0 ± 9.0	85.4 ± 6.2	90.7 ± 6.3
5×10^{-4} M DPN	79.6 ± 10.0	72.5 ± 7.3	77.6 ± 3.8
1×10^{-3} M Ascorbic Acid	81.0 ± 5.8	74.4 ± 11.1	78.0 ± 9.2
1×10^{-3} M 8-Hydroxyquinoline	84.2 ± 11.5	56.7 ± 7.7	57.9 ± 2.6
4×10^{-7} M Thyroxine	101.3 ± 7.3	93.1 ± 5.7	93.1 ± 8.0
4×10^{-5} M Thyroxine	101.8 ± 6.7	93.4 ± 5.7	93.5 ± 4.3
6×10^{-6} M KI	98.4 ± 13.7	90.1 ± 5.9	92.0 ± 5.4
6×10^{-4} M KI	10.1 ± 1.6	6.8 ± 2.2	6.1 ± 2.1
1×10^{-3} M $CuSO_4$	90.0 ± 28.6	71.8 ± 32.2	66.4 ± 23.3
1×10^{-3} M $ZnSO_4$	95.5 ± 18.7	88.6 ± 12.9	90.5 ± 9.3
1×10^{-3} M $FeSO_4$	95.3 ± 17.2	97.2 ± 12.9	90.6 ± 14.5
1×10^{-3} M $FeNH_4(SO_4)_2$	86.2 ± 14.6	96.2 ± 10.1	96.9 ± 3.4
1×10^{-3} M Na_2SO_4	99.7 ± 8.0	98.8 ± 7.9	99.3 ± 6.7

* $\%\ \text{Response} = \dfrac{\%\ \text{iodine uptake in the presence of reagent tested}}{\%\ \text{iodine uptake in unsupplemented buffer}}$.

These values based on six experiments for each reagent.

TABLE 7. EFFECT OF INCUBATION CONDITIONS ON IODINE UPTAKE

TSH m.u./ml.	% uptake I^{131}					
	Conditions					
	37° C 95% O_2-5% CO_2	37° C N_2	37° C Air	4° C 95% O_2-5% CO_2	4° C N_2	4° C Air
0	15.66	10.10	14.54	12.50	7.49	12.24
10	33.84	13.43	34.24	16.26	6.78	12.05
20	42.18	9.00	38.78	17.54	8.16	13.25

Iodine uptake was measured in media containing agents affecting enzyme activity and thyroid function. Two reactions were considered, first iodine uptake itself as seen in media unsupplemented with TSH and second, response to thyrotropic hormone. As seen in Table 6, the magnitude of standard deviations limited evaluation of all but a few of the reagents studied. 8-Hydroxyquinoline had little effect on iodine uptake but decreased response to thyrotropic hormone. Both uptake and stimulation by thyrotropin were impaired by DPN, ascorbic acid and 6×10^{-4} M KI. Influence of the other reagents on both of these phenomena was questionable because of the variety of responses of different minces. These responses reflected differences in tissue and were due to the reagent in question since the reactions were unaffected in solutions of equal molarity containing Na_2SO_4.

Iodine uptake in the presence and absence of thyrotropin appeared to depend on temperature and gaseous atmosphere during incubation. As seen in Table 7 replacement of 95% O_2–5% CO_2 with air had little effect on iodine uptake or response to thyrotropic hormone. In an atmosphere of nitrogen, uptake was reduced and response to tropic hormone disappeared. For these tissue reactions 37° C was more favorable than 4° C.

Responses of an isolated thyroid mince differed according to the weight of tissue employed. Iodine uptake of different amounts of thyroid tissue is seen in Table 8. With quantities greater than 800 mg., uptake was not appreciably increased in 10 m.u./ml. of thyrotropin. Such limitation of response may be due to depletion of I^{131} by tissue. Theoretical aspects of

TABLE 8. *In vitro* UPTAKE OF I^{131} BY THYROID MINCES AND THEIR RESPONSE TO TSH-EFFECT OF TISSUE WEIGHT

TSH m.u./ml.	% uptake I^{131}					
	Tissue weight, mg.					
	200	400	800	1000	1500	1800
0	8.8	14.4	24.4	30.3	35.2	36.8
10	27.1	35.0	49.1	52.0	48.0	53.9

Incubated in 4.0 ml. buffer at pH 7.4, 2 hrs. 37° C in 95% O_2-5% CO_2.

radioiodine distribution between tissue and incubation medium have been discussed by Freinkel and Ingbar (6).

Rate of iodine incorporation was studied by measuring % uptake at 30 minute intervals over a 3 hour incubation period. The flasks contained 0, 10 and 20 m.u./ml. of thyrotropin. Uptake rate curves of one experiment are seen in Figure 5. Four such experiments had two results in common: 1. The most rapid reaction occurred during the first 30 minutes. After this, rate was lower and represented removal of a constant portion of the unreacted I^{131} with variation of approximately $\pm 1\%$ in the absence of thy-

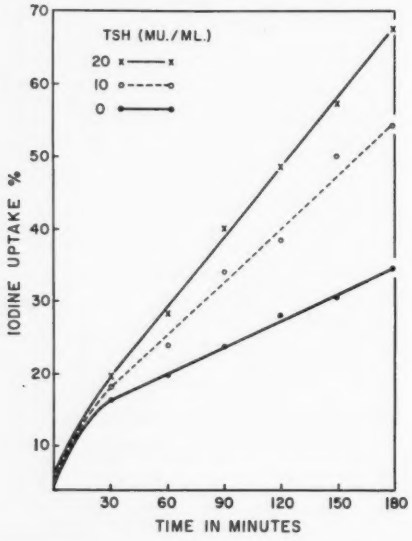

FIG. 5. Effect of thyrotropic hormone on rate of *in vitro* I^{131} uptake.

rotropic hormone and $\pm 10\%$ in its presence. 2. The initial velocity increased with addition of thyrotropin. In one experiment, in 0, 10 and 20 m.u./ml. of TSH, velocities of 0–30 min. were .4007, .4680 and .4797%/minute respectively.

DISCUSSION

Iodine uptake by thyroid tissue requires several enzyme systems catalyzing a series of consecutive reactions. With establishment of a steady state all reactions proceeded at a rate which depended on concentration of enzymes, activators and inhibitors in tissue. Alterations in these factors determined ultimately the curve representing effect of thyrotropic hormone on uptake. At low hormone levels a linear relation was observed when radioiodine uptake was plotted against logarithm of thyrotropin concentration. As TSH content was increased, there was a tendency for the curve to become sigmoid.

Absolute differences in uptake of tissue after 2 hours incubation in thy-

rotropic hormone permitted detection of the effect of thyrotropin on accumulation of both free iodide and protein bound iodine. As indicated by solubility in trichloroacetic acid, TSH increased I^{131} taken up as iodide if the radioiodide were added at the end of the incubation. When tissue was in contact with I^{131} during the entire incubation period, such accumulation of iodide was not apparent due probably to the formation of PBI^{131}. Thyrotropic hormone increased protein bound iodine of both tissue and incubation fluid. The latter illustrated a well established phenomenon associated with thyrotropic hormone—promotion of the discharge of protein bound iodine from the thyroid.

The values for iodine uptake reported in this study may be compared with those of other investigators who measured *in vitro* iodide utilization. Iodide uptake and release was studied by Bakke and Lawrence (3) who observed that uptake was greater and release was less from thyroid tissue incubated in media supplemented with propylthiouracil and thyrotropic hormone. Radioactivity released was analogous to the I^{131} of the wash solutions shown on Table 3 for flasks to which radioiodine was added at the beginning of the incubation. Tropic hormone decreased the I^{131} which could be removed on washing. The influence of blocking agents on the nature of the iodine uptake has been studied by Freinkel and Ingbar (7) who showed that I^{131} concentrated by mammary slices from media supplemented with tapazole was entirely soluble in trichloroacetic acid. Chromatographically it was identified as inorganic I^{131}. Elimination of tapazole resulted in the formation of significant amounts of trichloroacetic acid insoluble material. Taurog *et al.* (8, 9) also demonstrated on chromatograms the formation of protein bound iodinated compounds in slices and particulate fractions of the thyroid. The so-called iodine uptake reported in the present investigation represented protein bound iodine as well as trapped iodide. Bakke and Lawrence (3) found that collection of iodide under *in vitro* conditions was dependent on medium volume and carrier iodide concentration. To allow for variations in medium volume and tissue weight Freinkel and Ingbar (7) expressed iodine uptake from a tapazole containing medium as the concentration gradient S/M

$$\left(\frac{\text{counts/gm. of tissue}}{\text{counts/ml. of medium}}\right).$$

An analysis of the data on Table 3 has been carried out along similar lines in which the I^{131} mince/medium ratio has been expressed. In 0, 10 and 20 m.u./ml. of thyrotropic hormone values of 1.8, 7.6 and 11.2 were found when I^{131} was added at the beginning of the incubation period. If radioiodine was added at the end of the incubation little trichloroacetic acid precipitable iodine resulted. The mince/medium ratios in the corresponding concentrations of thyrotropic hormone were 0.48, 0.78 and 0.87. These values are not precisely comparable to the data reported by Freinkel and

Ingbar since no tapazole was added to the medium. However, they indicate that the values reported in this study for uptake are accompanied by a low but genuine error due to the iodide trapped because of the effect of the tropic hormone on osmoregulation. This error might be eliminated by amplifying the procedure to include a separation of the trichloroacetic acid precipitable iodine in tissue and incubation fluid. The authors declined routine use of this modification because it lengthened the assay period by one day.

Although thyroid tissue displays an ability to concentrate iodine, the nature and rate of reactions taking place depend upon conditions employed for study. After culturing thyroid glands *in vitro* for 25 days, Roche *et al.* (2) noted that the synthetic mechanism was not modified *in vitro* although under these conditions hormone formation was less intense, and following stimulation with thyrotropic hormone both triiodothyronines were formed in preference to thyroxine. Morton and Chaikoff (10) reported that incubation of slices of thyroid glands with NaI^{131} was associated with the production of diiodotyrosine and thyroxine. These conversions were reduced if tissue was minced and lost if tissue were homogenized. Taurog *et al.* (8) found that in whole homogenates there was formation of monoiodotyrosine but no diiodotyrosine. In our experiments, the physical form of tissue limited the response to thyrotropic hormone and iodide uptake. Uptake occurred when thyroid tissue was in any state of subdivision but response to thyrotropic hormone was lost when tissue organization was disrupted by homogenization or freezing. Such responses of thyroid tissue suggest an association of these reactions with the distribution of I^{131}. Microscopic examination of the tissue employed indicated that the mince differed little from a section of an intact thyroid gland. Both showed regular thyroid follicles filled with colloid and lined by cuboidal epithelium. Thyroid homogenates showed an occasional isolated follicle against a background of amorphous fragmented eosinophilic material and debris. A frozen slice contained follicles characterized by the absence of colloid.

Sensitivity of thyroid tissue to thyrotropic hormone under *in vitro* conditions has been studied. Bakke and Lawrence (11) found that iodine collection of slices was stimulated by as little as 1×10^{-2} m.u. of TSH whereas 8 m.u. were required for organic binding. As reported by Lawrence and Bakke (12) water transfer of thyroid slices was altered by 8×10^{-5} u./ml. Using tissue culture techniques, Bottari (13) measured iodine uptake of portions of thyroid glands and detected 1×10^{-5} I.U. This figure converted to U.S.P. units with factors summarized by Overbeek *et al.* (14) is equivalent to .05 U.S.P. m.u. An *in vitro* technique was reported by Florsheim *et al.* (15) who found that incorporation of P^{32} in phospholipids of thyroid slices was regulated by TSH with a linear response between 2 and 200 m.u./ml. In our experiments, there was detection of at least 1 to 2 m.u./ml. of thyrotropic hormone. All glands were not equally responsive to TSH,

and sensitivity was reflected in the slope of the regression line. The steeper curves relating response to dose provided a more precise estimate of different concentrations of thyrotropin and also permitted the detection of lower levels of TSH. In order to increase the accuracy of measuring thyrotropic hormone in this *in vitro* system, an arbitrary standard was employed in evaluating alterations in iodine uptake. A thyroid mince was expected to display a difference of 10% in uptake between 0 and 10 m.u./ml. of thyrotropic hormone. In a group of 68 minces, 60 met such a standard indicating that 88% of the minces displayed satisfactory response to TSH. Because of the uniformity of response of beef thyroid minces, it is suggested that this procedure may be applicable as an *in vitro* assay for thyrotropic hormone. Although not maximally sensitive, the technique is simple, rapid and accurate. It minimizes variability of thyroid tissue and is not affected by thyroxine.

REFERENCES

1. Botkin, A. L., C. D. Eskelson, H. E. Firsheim and H. Jensen: *J. Clin. Endocrinol. and Metab.* **14**: 1219. 1954.
2. Roche, J., M. Pavlovic and R. Michel: *Biochim. biophys. acta* **24**: 489. 1957.
3. Bakke, J. L. and N. Lawrence: *Endocrinology* **58**: 531. 1956.
4. Bottari, P. M.: *Arch. internat. physiol.*, Liege **64**: 117. 1956.
5. Joftes, D. L. and S. Warren: *J. Biol. Photogr. Ass.* **23**: 145. 1955.
6. Freinkel, N. and S. H. Ingbar: *J. Clin. Endocrinol. and Metab.* **15**: 442. 1955.
7. Freinkel, N. and S. H. Ingbar: *Endocrinology* **58**: 51. 1956.
8. Taurog, A., G. D. Potter and I. L. Chaikoff: *J. Biol. Chem.* **213**: 119. 1955.
9. Taurog, A., W. Tong and I. L. Chaikoff: *J. Biol. Chem.* **227**: 759. 1957.
10. Morton, M. E. and I. L. Chaikoff: *J. Biol. Chem.* **144**: 565. 1942.
11. Bakke, J. L. and N. Lawrence: *Clin. Res. Proc.* **II**: 85. 1954.
12. Lawrence, N. L. and J. L. Bakke: *Biochim. biophys. acta* **19**: 196. 1956.
13. Bottari, P. M.: *Ciba Foundation Colloquia on Endocrinology* **11**: 53. 1957.
14. Overbeek, G. A., J. Fokkens, A. Querido, J. de Visser and P. Canninga: *Acta endocr.* **14**: 285. 1953.
15. Florsheim, W. H., N. Moskowitz, J. R. Schwartz and M. E. Morton: *Endocrinology* **60**: 683. 1957.

THE POSSIBLE ROLE OF OXYTOCIN IN STIMULATING THE RELEASE OF PROLACTIN[1]

S. M. McCANN, ROBERT MACK AND C. GALE

Department of Physiology of the University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania

ABSTRACT

Hypothalamic lesions in the supraoptico-hypophysial tract of lactating rats produced a block of both milk ejection and milk secretion. The defect in milk secretion was correlated with the deficiency in endogenous oxytocin secretion, but there was no correlation between polydipsia and milk ejection or milk secretion. The effective lesions interrupted the supraoptico-hypophysial tract either in the median eminence or in front of this structure. The defect in milk secretion was seen in rats where median eminence, hypophysial portal vessels, and anterior lobe of the pituitary appeared to be essentially normal on histological examination. Pitocin and synthetic oxytocin delayed mammary involution in lactating rats. Pitressin produced a smaller effect. The effect of Pitocin and synthetic oxytocin persisted in rats with hypothalamic lesions. Since a defect in endogenous oxytocin release led to a defect in milk secretion, whereas oxytocin delayed mammary involution, it was postulated that oxytocin may be the neurohumor stimulating the release of lactogenic hormone.

PREVIOUS work by a number of investigators has indicated that vasopressin or a closely related polypeptide may play a role in the release of adrenocorticotrophin (ACTH) (1, 2, 3). In view of this relationship, it became of interest to speculate on a possible role of the other neural lobe hormone, oxytocin, in regulating anterior pituitary function. Andersson (4), and Cross and Harris (5) demonstrated that suckling produces a reflex release of oxytocin which in turn causes milk ejection. Selye (6) and others (7, 8) have further shown that suckling stimulates milk secretion by a reflex augmentation of the release of lactogenic hormone. The principal evidence for this view is the demonstration that mammary involution is delayed by the continued suckling of breasts from which the egress of milk has been prevented. Since lactogenic hormone has a similar effect in delaying mammary involution, Hooker and Williams suggested that suckling must stimulate a discharge of this hormone (9). Because suckling releases both oxytocin from the neurohypophysis and lactogenic hormone from the anterior lobe, it is reasonable to postulate that the release of oxytocin may stimulate the release of lactogenic hormone. This suggestion was apparently first made by Peterson (10). Subsequently, Benson and Folley reported that oxytocin delays the involution of the

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breast which follows weaning (11), which provided the first experimental evidence for the postulated role of oxytocin in the release of lactogenic hormone.

The present experiments were designed to test the hypothesis that oxytocin triggers the release of lactogenic hormone in the rat. In the first series of experiments, the effect of hypothalamic lesions designed to prevent the secretion of oxytocin was studied. Then, the effect of exogenous oxytocin on the course of mammary involution was determined in normal rats and those with hypothalamic lesions. A defect in milk secretion as well as milk ejection was demonstrated in the rats with lesions, whereas exogenous oxytocin delayed mammary involution in both normal rats and those with lesions. A preliminary report of this work has already appeared (12).

METHODS

Lactating Wistar rats were used throughout this study. They were fed a diet of Purina chow supplemented with oranges and bread.

Milk secretion was estimated in most cases simply by recording to the nearest 0.1 gm. the daily weight gain of the litter, and then converting this to a rate of gain by expressing it in mg. gained/gm. of weight of litter/hour. Using data of Brody (cf. 13) it is possible to estimate milk yield by multiplying gain of body weight by 100/55.

Where oxytocin was injected to cause milk ejection, a direct measure of milk yield was attempted by weighing the litter before and $\frac{1}{2}$ to 1 hour after oxytocin administration.

In rats with failure of oxytocin release where litters were suckled on alternate days by normal mothers, the calculation of total milk flow of deficient mothers was more complicated. Two different methods of calculation were used and compared—one based upon daily weight gain of the litter, the other upon gain of the litter following oxytocin injection into the deficient mother. These calculations are more fully described in the appropriate section of the results.

A. *Lesion experiments:* On the second day of lactation each litter was reduced to six members. If the litter was gaining weight hypothalamic lesions were produced in the mother on the fourth day of lactation, though in some instances they were made later, i.e., as late as the eighth day. Lesions were produced with the Krieg stereotaxic instrument with the rat under ether anesthesia as described previously (1). Coordinates were used which had been shown to produce lesions that interrupted the supraoptico-hypophysial tract either in the median eminence or in front of this structure. "Large" lesions were made by coagulating bilaterally with a current of 6 mA. for 15 seconds, whereas "small" lesions were made with a current of 2.4 mA. for 5 seconds. After a two-hour recovery period, the mothers were returned to their litters. In rats with median eminence lesions, where a defect in milk ejection was thought likely to occur, an injection of commercial oxytocin (Pitocin, 0.75 Units (U.) diluted to 0.7 ml. with physiological saline, intraperitoneally (I.P.)) was given several hours after the operation. Thereafter, whenever the litters nursing mothers with lesions lost weight overnight, these mothers were injected 1–3 times a day with oxytocin beginning that morning. If lactation was interrupted following placement of the lesions in spite of oxytocin injections, the litters of the operated mothers were exchanged with those of foster mothers every 24 hours. In this way continued suckling of the rats with lesions was assured, and litters could be kept in good condition, though somewhat subnormal in weight, throughout the experiment.

In order to provide control data for these experiments, normal mothers were isolated from their litters overnight and then anesthetized with Nembutal (4 mg./100 gm., I.P.). The milk obtained by the litters from the anesthetized rats before and after the injection of oxytocin was then determined by weighing the litter.

Water intake and body weight of the mother rats with lesions were recorded at frequent intervals. At autopsy, the breast tissue was examined grossly, and in some instances histologically after fixation in 10% formalin, paraffin imbedding, sectioning at 5μ, and staining with hematoxylin and eosin. The right ovary and left adrenal were dissected free of fat and weighed to the nearest 0.1 mg. on a torsion balance. Uteri were examined but were not weighed. Brains with pituitaries attached were preserved in either formalin or Bouin's solution. Diencephalon and pituitary were serially sectioned at 5 or 15μ, and every 5th or 10th section mounted. Diencephalons were stained with either Luxol fast blue (14) or the chrome-alum hematoxylin phloxine method of Gomori (15). Pituitaries were stained with either hematoxylin-eosin, azo-carmine, or chrome-alum hematoxylin phloxine.

B. *Injection experiments:* To study the effect of possible neurohumors upon lactation in mothers not permitted to suckle their young, litters were again reduced to 6 members on the 2nd day of lactation and removed on the 4th day. All mother rats were then injected, I.P., thrice daily for 9 days with 0.3 ml. of either physiological saline, Pitocin, Pitressin, or synthetic oxytocin (Syntocinon[2]). Hypothalamic lesions were made in certain rats as described for "small" lesions, immediately after removal of their litters. The remainder had no hypothalamic injury. At sacrifice, the left abdominal breast was examined grossly and histologically after preparation as described under A.

RESULTS

I. The Effect of Hypothalamic Lesions on Milk Ejection and Lactation in the Rat

A. *Normal Rats.* Five mother rats gave milk at such a rate that their litters gained an average of 3.7 mg./g. of litter/hr. during the first 2 weeks of lactation (Table 2). This rate of gain served as the control value for rats with lesions where a foster mother was not required. The foster mothers, which were used if lactation was interrupted after lesions, gave milk at a more rapid rate, providing an average weight gain by each litter of 5.6 ± 0.4 mg./g./hr. This was probably due to the fact that under these conditions the mother was supplying most of the milk for 2 sets of litters of 6 infants each. The increased suckling stimuli apparently resulted in a greater milk yield by the mother. These mothers were considered the controls for rats with lesions whose litters required foster mothers.

Litters which were isolated from their mothers lost weight at a rate of 2.8 ± 0.17 mg./g./hr. When placed with Nembutal-anesthetized mothers which had been isolated overnight to allow milk to accumulate, the litters were able to obtain no milk (Table 1), indicating that anesthesia completely blocks milk ejection and that no milk is obtained by passive withdrawal from breasts containing milk in the rat. This confirms the extensive observations of Grosvenor and Turner (16). Since no milk is obtained in

[2] Syntocinon was kindly supplied by Dr. S. Gimpel of Sandoz Co., Hanover. New Jersey.

the absence of oxytocin, weight change of a suckling litter in the absence of injected oxytocin has been considered an index of the degree of failure in endogenous oxytocin secretion in rats with lesions. Of course if no milk is present for ejection, the index is useless.

The injection of oxytocin in these anesthetized rats produced a rapid milk ejection and permitted the litters to obtain milk (Table 1). The maximum amount obtained under such conditions provides an estimate of the capacity of the litter to ingest milk after isolation and a single injection of oxytocin. This maximum value was obtained by isolating foster mothers overnight since they might be expected to accumulate more milk than normal mothers. It appears that the litter which has been fasted for a period of 15 hours can ingest at least 9–10% of its body weight in milk after a

TABLE 1. THE EFFECT OF ANESTHESIA ON MILK EJECTION IN THE RAT

Rat No.	Weight gain of litter (grams) during 30 minute periods		
	Isolated	Suckling a mother rat under nembutal	
		No oxytocin	Oxytocin*
1	−0.1	−0.1	1.6
2	−0.15	−0.1	2.5
3	0.0	−0.3	1.7
4	−0.2	−0.3	5.6
Mean	−0.1	−0.2	2.8

* 0.75 I.U. Pitocin given intraperitoneally.

single injection of oxytocin has been given to the anesthetized foster mother. The mean litter weight gain in 5 such experiments was 9.2% of body weight. This measurement provides information concerning the maximum weight gain of litters which might be expected after an injection of oxytocin into a mother with a deficit of endogenous oxytocin release.

B. *Rats with Lesions Which Did Not Block the Release of Oxytocin.* The results obtained in 14 rats where the lesion did not prevent the infants from obtaining milk are illustrated in Table 2. As indicated in the table, these lesions were in various hypothalamic areas. Most lesions appeared to produce a slight decrease in weight gain of litters when compared with litters from control, non-operated mothers. The mean weight gain of all rats was 9% less than that of control litters (Fig. 3). It was noted that most animals gave very little milk on the first post-lesion day, and this accounts for much of the decrease in weight gain of these litters. The remainder of the slight deficiency in these rats may be accounted for by the slight weight loss of the mothers, suggesting a decrease in food intake, and by the fact that, in some cases some damage was certainly sustained by the supra-optico-hypophysial tract. Such damage is shown in section C to produce an alteration in milk secretion. Because Olivecrona (17) has found that destruction of the paraventricular nuclei results in a decrease in neural

lobe oxytocin content, lesions were placed in an attempt to destroy the paraventricular nuclei in 5 cases (Table 2, E, paraventricular region). These nuclei sustained severe damage in 4 rats with a cell loss estimated to be 50–75%; in the 5th rat (P-7), however, a nearly complete destruction of the paraventricular nuclei was achieved (estimated cell loss > 95%). The observation that there was little or no impairment of lactation in

TABLE 2. RESULTS IN RATS WITH LESIONS WHICH FAILED TO BLOCK LACTATION

Rat No.	Mean litter weight gain (mg./g./hr.)	% of control[1]	Right ovarian wt. (mg.)	Left adrenal wt. (mg.)	% change in body weight of rat	H_2O intake (ml./day)	Size lesion
A. Preoptic Area							
12	2.7	85			−17	N[2]	L[3]
B. Dorsal Hypothalamus and Ventral Thalamus							
CL7	3.0	89			−16	16	S[3]
CL6	4.0	105			−15	22	S
CL5	2.2	77	17	19	− 8	28	S
1C	2.6	83	27	23	− 7	34	S
CL3	3.0	89	29	28	+ 9	50	S
Mean	3.0	89			− 7	30	
C. Lateral to Median Eminence							
4	3.0	89	32	39	− 9	34	S
6	2.8	86	50	63	− 9	45	S
D. Posterior to Median Eminence							
2	2.8	86	30	52	− 2	N	S
3	2.8	86	47	57	− 9	N	S
E. Paraventricular Region							
P-1	3.8	102	55	34	− 1	91	S
P-2	4.0	105	51	37	−14	48	S
P-3	2.5	82			− 2	40	S
P-4	3.7	100			−16	62	S
P-7	3.9	103	37	43	− 5	43	M[3]
Overall Mean:	3.1	91	38	39	− 8		
Mean of 5 controls	3.7	100					

[1] For method of calculation, see page 876.
[2] Approximately normal.
[3] L = "Large" lesions; M = "Medium" lesions; S = "Small" lesions.

these cases can be considered as evidence against an essential role of these nuclei in oxytocin release or lactation.

Two of the rats with lesions in the paraventricular nuclei exhibited a mild polydipsia which is attributed to the fact that the lesions in both cases extended to the base of the brain so as to produce an injury to the supraoptico-hypophysial tract. In neither case was this injury sufficient to cause a block of milk ejection or secretion.

C. *Rats with Lesions Which Interfered with the Release of Oxytocin.* Lesions designed to interrupt the supraoptico-hypophysial tract, and thereby

prevent the release of oxytocin, produced a different picture (Table 3A). In these cases the infants were able to obtain little or no milk despite vigorous suckling. They lost weight when left with the mother rat, which was taken to mean that the milk ejection reflex was interrupted by the deficiency of endogenous oxytocin secretion. These rats were enabled to obtain a certain amount of milk by periodic injections of oxytocin, thus confirming the earlier findings of Cross and Harris (5) in the rabbit and Benson and Cowie (18) in the rat.

TABLE 3A. LACTATIONAL PERFORMANCE OF RATS WITH LESIONS IN THE SUPRAOPTICO-HYPOPHYSIAL TRACT

Rat No.	Mean litter weight gain (mg./g./hr.)			% of control*	Right ovarian wt. (mg.)	Left adrenal wt. (mg.)	% change in body weight of rat	H_2O intake (ml./day)
	Without oxytocin	After oxytocin	Total					
1. "Large" Lesions								
4	−3.1	+0.6[1]	−2.5	4	37	25	− 7	200
1D	−3.1	+2.3[3]	− .8	24	40	35	−10	110
2D	−2.1	+1.7[3]	−0.4	29	45	40	−12	65
4D	−3.5	+1.3[3]	−2.2	7	34	27	0	35
Mean:	−3.0	+1.5	−1.5	16	39	32	− 7	102
2. "Small" Lesions								
2B	−3.3	+1.7[1]	−1.6	14	46	24	+13	24
5B	−3.1	+3.2[1]	+0.1	34	44	39	−12	150
6B	−3.5	+2.7[1&3]	−0.8	24	32	54	−16	85
7B	−4.3	+1.8[1&3]	−2.5	4	45	35	− 8	35
8B	−3.4	+2.1[1&3]	−1.3	18	47	48	−13	37
A5	−3.1	+2.2[3]	−0.9	23	37	45	−14	170
A4	−1.8	+1.8[3]	0.0	33	55	40	− 6	84
Mean:	−3.2	+2.2	−1.0	21	40	41	− 8	84
3. Partial Lesions								
9B	−2.1	+4.2[3]	+2.1	75	37	53	− 9	240
5	−1.9	+2.9[3]	+1.0	58	47	41	− 8	40

* For method of calculation see page 876.
[1] One injection of oxytocin per day.
[3] Three injections of oxytocin per day.

The marked reduction in milk secretion in these rats made it necessary to employ foster mothers for the infants. Litters were switched back and forth daily between the two mothers so that suckling stimuli were maintained at all times. The routine consisted of switching the litters one hour after the deficient mother received her morning oxytocin injection; thus, the hungry infants which had been unable to obtain milk overnight from the rat with lesions were enabled within this hour to obtain the milk that had accumulated since the last injection. Because the litter was switched to the foster mother after this large intake of milk, it was impossible to use the simple recording of litter weight as an index of lactation in the rats with these effective lesions. Instead, the sum of the weight gains during a 24 hour period, following the individual oxytocin injections, expressed as

mg./g. of litter/hour was determined. Then, the weight change of the litter overnight in the absence of injected oxytocin was calculated as mg./g. of litter/hour. (This was taken as an index of the ability of the infants to obtain milk by secretion of endogenous oxytocin since it has been shown that rats do not obtain milk by passive withdrawal from the breast.) The sum of the weight gains occurring after oxytocin injections was added to that obtained in the absence of the drug to give the total weight gain of the litter. This was then compared with the average weight gain of each litter when nursing the control foster mother to determine the percentage deficit in lactation.[3] This method overestimates the lactational performance of the rats with lesions since all milk obtained after injection of oxytocin is not converted to tissue. In two cases where it was necessary to use oxytocin but a foster litter was not required (Table 3, Partial lesions), the actual weight gain of the litter was compared with the calculated gain using the above method. It was found in both instances that the calculated weight gain was greater than the actual gain; the cumulative error at the end of 11 days was 28 and 32%. In spite of this error it is felt that this method is a useful one and that it gives values which are closely related to lactational performance.

An alternative method of comparison of the lactational performance of rats with lesions with that of the foster mothers is to convert the weight gain of the litters nursing foster mothers into an estimate of milk yield. According to Brody (cited by Cowie and Folley (13)), the weight gain of a litter is roughly equivalent to 55% of the milk yield. Consequently an estimate of the milk yield can be made by multiplying the litter gain by 100/55. This value, which for the foster mothers is 10 mg./g./hr., can then be compared with the milk yield obtained after oxytocin in rats with effective lesions. A good estimate of the percentage reduction in milk yield in rats with lesions results since these rats give almost no milk during the periods when oxytocin is not given. Both methods of calculation of the lactational performance of the rats with lesions agree surprisingly well.

"Large" lesions in the supraoptico-hypophysial tract caused the litters to lose weight at a rate of 3.0 mg./g./hr. when oxytocin was not administered (Table 3A). Oxytocin injections permitted a weight gain at a rate of 1.5 mg./g./hr. Thus, in spite of injections of oxytocin which permitted removal of milk from the mothers, the litters lost weight at a rate of 1.5 mg./g./hr. When these litters were suckling foster mothers on alternate days, they gained weight at a rate of 5.6 mg./g./hr. Thus, in these rats there was a deficiency of both milk ejection (gain without oxytocin) and

[3] The method of calculation was as follows: if when suckling the foster mother the litter gained weight at a rate, x, greater than that of the isolated litter, whereas when suckling the rat with lesions it gained at a net rate of y greater than that of the isolated litter, then the lactational performance in percentage of normal of the rat with lesions would be $(y/x) \times 100$.

milk secretion (total weight gain). The latter was estimated to be only 16% of normal (Table 3A, Fig. 3). This marked reduction in milk secretion was reflected in a decreased size and partial involution of breast tissue when examined grossly and microscopically. The complete course of the experiment in one of these cases is shown in Figure 1. It is apparent that little milk was obtained in the absence of oxytocin and that, even when it was given, the milk yield was grossly subnormal. The greatest amount of milk was obtained on the first post-operative day. This was followed by a gradual decrease in milk yield to a minimum value on the 5th postopera-

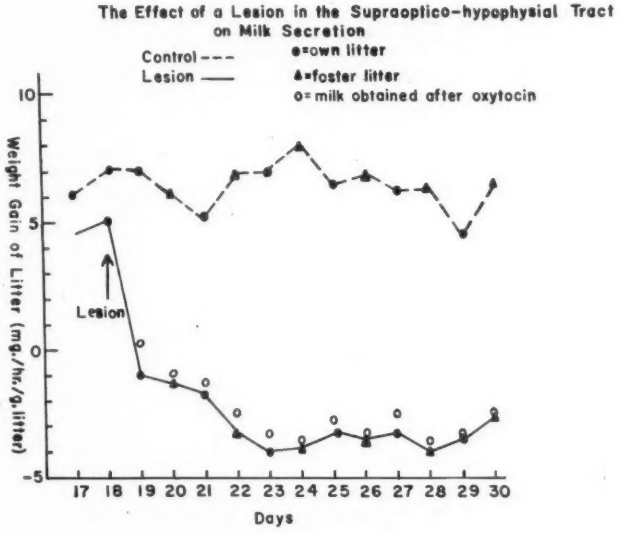

FIG. 1

tive day. In most other cases the maximum milk yield also occurred in the immediate post-operative period.

"Small" lesions (Table 3A) produced a similar picture, but the reduction in milk production appeared to be not quite as pronounced, the mean secretion being 21% of normal (Table 3A, Fig. 3). The breasts of these animals exhibited less involution than that seen in rats with "large" lesions. The results obtained in one of these cases are illustrated in detail in Figure 2. It is noteworthy here that, after an initial decline in litter weight gain, there was a rebound toward normal from the third to the seventh day, by which time the milk yield had once again fallen to very subnormal levels, where it remained for the remainder of the experiment. This rebound increase in milk yield for a short time during the first post-operative week, noted also in rats with large lesions, was seen altogether in 7 out of the 13 cases with a deficiency of oxytocin release.

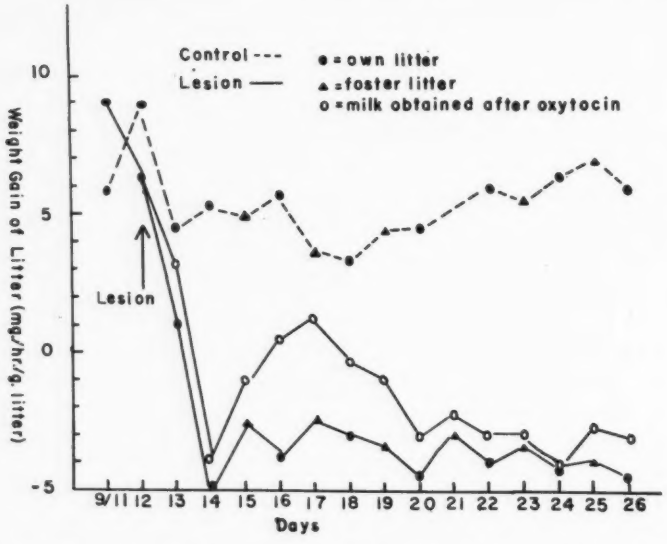

FIG. 2. The effect of a "small" lesion in the supraoptico-hypophysial tract on milk secretion

It is surprising that the weight loss in the absence of oxytocin in litters suckling rats with lesions often exceeded that found when the litters were isolated and no intake occurred (Tables 1 and 3A). This discrepancy may be related to a higher environmental temperature when with the mother, which produced a greater insensible loss of water. Exercise during suckling efforts may have also contributed to a greater weight loss. Lastly, diuresis induced by ingestion of milk following the preceding injection of oxytocin would increase the rate of weight loss of the litter. This factor is relatively small since the amount of milk obtained following the last oxytocin injection was usually very small.

A relationship between endogenous antidiuretic hormone (ADH) release and ACTH secretion has been demonstrated in rats with hypothalamic lesions (19). This later relationship was obtained by plotting water intake as an index of the severity of diabetes insipidus and thereby deficiency of ADH versus ACTH release. An attempt to relate water intake

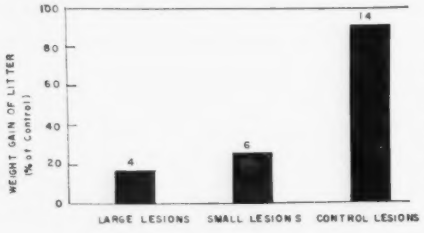

FIG. 3. The Effect of Hypothalamic Lesions on the weight gain of litters. The height of each bar represents the mean effect of each type of lesion. The number above each bar represents the number of mother rats with that type of lesion.

to milk secretion is shown in Figure 4, which illustrates that in the present experiments there was no correlation between the presence of a deficiency of ADH release, as indicated by polydipsia, and milk secretion. Rats with a near maximal impairment in milk secretion were seen to have no diabetes insipidus, whereas other animals with definite diabetes insipidus had essentially normal milk secretion. Furthermore, it is equally apparent (Tables 2 and 3) that milk ejection (i.e., weight gain in the absence of oxytocin) was also unrelated to polydipsia.

There was no correlation between the deficit in lactation and ovarian or adrenal weights, though occasionally these weights were definitely subnormal (Tables 2 and 3). Impaired lactation was likewise seen in the presence of normal sized uteri. Furthermore, since the weight loss which was

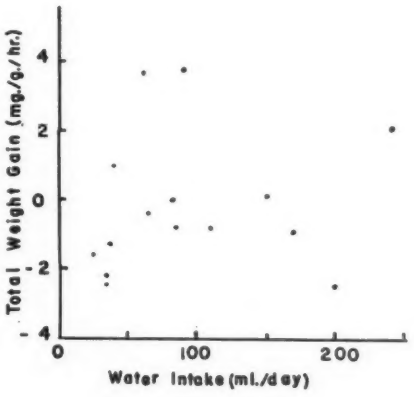

FIG. 4. The failure of litter weight gain to correlate with water intake, indicating that milk secretion in the rat with hypothalamic lesions is unrelated to diabetes insipidus.

often seen in mothers after lesions interrupting lactation was no greater than that seen after control lesions, it appears that changes in nutritional status cannot account for the results.

Serial sections of diencephalon and pituitary were made in all rats with marked impairment in lactation (Table 3B), and in all but 2 of the 14 control rats. In all cases where a defect of milk ejection and milk secretion was present the lesions were seen to produce at least a partial interruption of the supraoptico-hypophysial tract as judged by their location, by an atrophy of the infundibular process, and by a decrease in the number of supraoptic neurons (Table 3B). In 2 cases where it was studied, there was a disappearance of neurosecretory material from the infundibular process. In one of the two cases (Rat 5) where a less complete impairment of milk ejection and lactation was seen, it was apparent from the sections that the interruption of this tract was less complete than in the other cases. This was not surprising in this case since the lesions had been designed to reach the base of the hypothalamus just lateral to the supraoptico-hypophysial tract. However, in rat 9B the other rat with a less pronounced deficit, the

supraoptico-hypophysial tract had sustained considerable injury. A less complete injury to this tract did not block milk ejection or lactation in rats P-1, P-4, 6, 4 and 12 (Table 2). It is of interest that preliminary observations suggest that loss of neurosecretory substance from the neural lobe appears to be a very sensitive index of injury to the supraoptico-hypophysial tract in lactating rats since the material was depleted from 6 neural lobes where the injury to this tract was minimal and lactation was not blocked, whereas in 6 rats where the tract was spared, there was abundant

TABLE 3B. LOCATION OF LESIONS IN RATS WITH INJURY TO THE SUPRAOPTICO-HYPOPHYSIAL TRACT

Rat No.	Structure injured (graded as to severity from + to ++++)						
	Median eminence, M.E.	Portal in M.E.	Veins in stalk	Supra-optic nucleus[1]	Paraven-tricular nucleus[2]	Atrophy of neural lobe	Supra-optico-hypo-physial tract
1. "Large" Lesions							
4	++++	++++	0	+++	++	++	+++
1D	+++	+++	0	++	++	++	++
2D	+	+	0	++	+++	++	++
4D	++	+++	0	++	+++	+	++
2. "Small" Lesions							
2B	+	+	0	+++	+++	+++	+++
5B	++	+	0	+++	+	− − −	+++
6B	+++	+++	0	++	++	++	+++
7B	+	+	0	+++	++	− − −	+++
8B	+	+	0	++	++	++	++
A5	+++	+++	0	++++	++	+++	+++
A4	+	+	0	++	+	+	++
3. Partial Lesions							
9B	++	++	0	++	+	++[4]	++
5	0	0	0	+−++[3]	+−++	+[4]	+−++

[1] Injury restricted to retrograde degeneration of neurons.
[2] Direct injury from electrode tracts, and retrograde degeneration.
[3] Almost complete unilateral destruction.
[4] Complete loss of neurosecretory material.

neurosecretory material in the neural lobe. Apparently, in the presence of injury to the tract reducing the reserve of secretory tissue, function may be maintained but at the expense of storage of neurosecretory material.

In the rats with deficient oxytocin release and milk secretion, the interruption of the supraoptico-hypophysial tract occurred either in the median eminence or just in front of this structure. In 7 rats it was damaged more or less severely, while in 6 rats the eminence appeared to be completely, or nearly completely, intact. It, therefore, appears that injury to the median eminence itself or structures in its immediate vicinity is not a necessary condition for a defect in lactation. Specifically, it is unlikely that injury to fiber tracts entering the eminence from the posterior hypothalamus contributed to the effect or that injury to portal vessels, pars tuberalis, or arcuate nuclei played a role. A section through the intact median eminence of rat No. 7 B is shown in Figure 5. Transverse sections through the hy-

pothalamus of 4 of the rats in which the median eminence was spared are illustrated in Figure 6.

Study of the anterior lobes of the rats with lesions revealed that the vascular supply to the gland was abundant and no infarcts were seen.[4] In one case, rat 4 (Table 3A), the anterior lobe was definitely smaller than normal whereas in the other cases it appeared to be about as large as that of the control rats; however, no volume determinations have as yet been

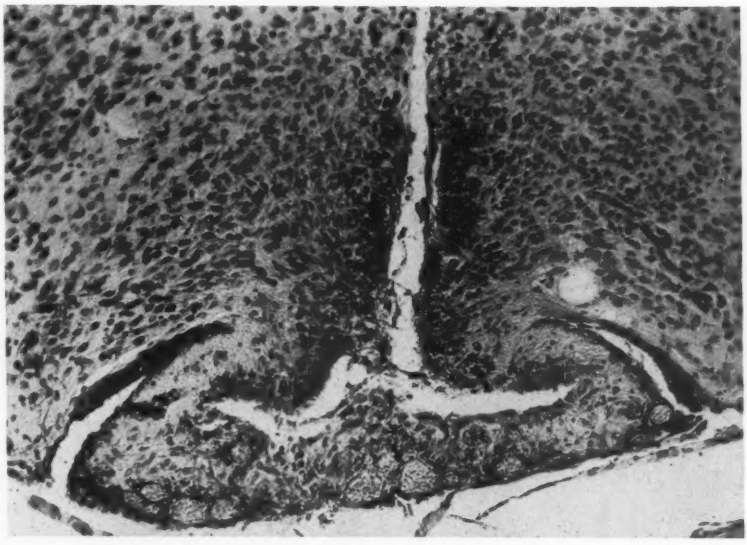

FIG. 5. A transverse section through the median eminence of one of the rats with a "small" lesion in the hypothalamus demonstrating intact hypophysial portal veins filled with red cells.

made. All anterior lobes contained eosinophiles, basophiles, and chromophobes, but no attempt has been made to determine if abnormalities in the numbers of these forms were present.

II. The Effect of Posterior Lobe Extracts on the Course of Mammary Involution in the Rat

A. *Normal Rats.* After removal of the litter, the rat's mammary glands involute. There is a failure of secretion, followed by a loss of alveolar

[4] In earlier experiments with lesions located more posteriorly in the median eminence, infarcts similar to those seen after stalk section were noted (21). These partiuclar rats with lesions also had considerable apparently well vascularized pituitary tissue and demonstrated lactational findings like those of rats with the "large" lesions reported here. Because of the question as to the state of anterior lobe vascularization, these rats were not included in the present report.

structure, and a conversion of parenchyma into fat (6, 11). This process is nearly complete nine days after removal of the litter in the saline injected rats (Table 4). In contrast, injections of Pitocin, a commercial extract of oxytocin, were able markedly to retard the involution. There was much more parenchymal tissue in the mammae from oxytocin-injected rats. Furthermore, there was a considerable preservation of alveoli, and secretion was present. In some cases alveoli and ducts were abnormally distended with secretory material; however, these glands were usually not in a lactational condition. The effect was then a retardation but not a prevention of involution. The minimal effective dose of Pitocin to produce a retardation of involution appeared to be about 0.25 U., I.P., three times daily.

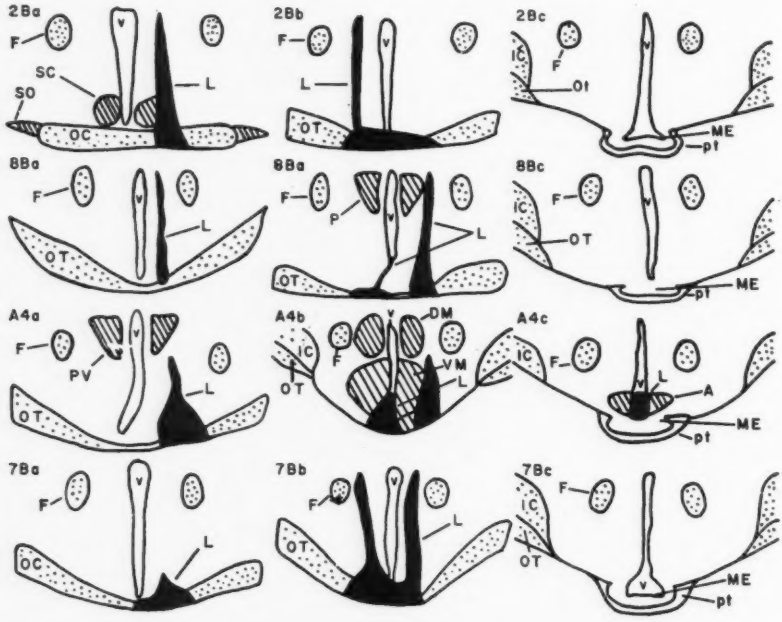

FIG. 6. Drawings of transverse sections through the hypothalamus of four of the rats with "small" lesions. Sections marked "a" pass through the rostral tip of the lesions; those marked "b" pass through the greatest extent of the lesions; and those marked "c" are just caudal to or at the caudal tip of the lesions.

Key for abbreviations:

IC—Internal capsule
CC—Optic chiasm
OT—Optic tract
F—Fornix
V—Third ventricle
A—Arcuate nucleus
DM—Dorsomedial nucleus
PV—Paraventricular nucleus
SC—Suprachiasmatic nucleus
SO—Supraoptic nucleus
ME—Median eminence
pt—Pars tuberalis of pituitary
L—Lesions

Pitressin, a commercial extract of vasopressin, produced a definite though less marked effect (Table 4). Its potency appears to be roughly half that of oxytocin, though more work is required to establish this. Examples of the effects of Pitocin and Pitressin are shown in Figures 7 and 8. Syntocinon, a preparation of synthetic oxytocin, appeared to be as active as Pitocin in inhibiting mammary involution (Table 4).

B. *Rats with Hypothalamic Lesions.* Since oxytocin produces milk ejection by inducing contraction of myo-epithelial elements in the breasts, it

TABLE 4. RETARDATION OF INVOLUTION OF THE BREAST OF RATS PRODUCED BY POSTERIOR LOBE EXTRACTS

Treatment	Response[1]	
	Individual	Mean
A. *Normal Rats*		
1. Saline	0, 0, 0, 0, 1, 0, 0, 0, 0, 1	0
2. Pitressin,		
1.0 U.,[2] t.i.d.[3]	1, 2, 2, 2, 2, 1	2−
0.5 U.,[2] t.i.d.[3]	0, 1, 2, 2	1
3. Pitocin,		
1.0 U., t.i.d.	2, 2, 3, 1, 2, 4, 2	2+
0.5 U., t.i.d.	1, 3, 2, 2	2
0.25 U., t.i.d.	1, 1, 2, 0	1
4. Synthetic Oxytocin		
1.0 U., t.i.d.	2, 2, 2, 1, 3	2
B. *Rats with Hypothalamic Lesions*		
1. Saline	1, 1, 2, 1, 2, 1 2, 1, 2, 0, 1, 2	1
2. Pitocin		
1.0 U., t.i.d.	3, 4, 2, 3, 1, 3	3−
3. Synthetic Oxytocin		
1.0 U, t.i.d.	4, 3, 4, 4, 2, 3, 3	3

[1] Responses graded from 0–4; 1–3 = progressively decreasing degrees of involution; 4 = lactational condition.
[2] U. = International unit.
[3] t.i.d. = Thrice daily.

was possible that this contraction produced afferent impulses which would reflexly cause stimulation of lactogenic hormone release by an unknown mechanism and thereby a retardation of involution of the breast. A reflex type of retardation has already been demonstrated to be produced by suckling or application of turpentine to nipples (6, 21). It seemed of interest to determine if oxytocin could still retard mammary involution in the presence of a hypothalamic lesion. Although the mammary involution in the saline injected rats with hypothalamic lesions was not as complete as seen in the normals (Table 4), mammary involution was significantly retarded by either Pitocin, or synthetic oxytocin (Table 4, Fig. 9). Serial sections of hypothalamus and pituitary of the seven rats injected with synthetic oxytocin revealed that all of these lesions were similar to those

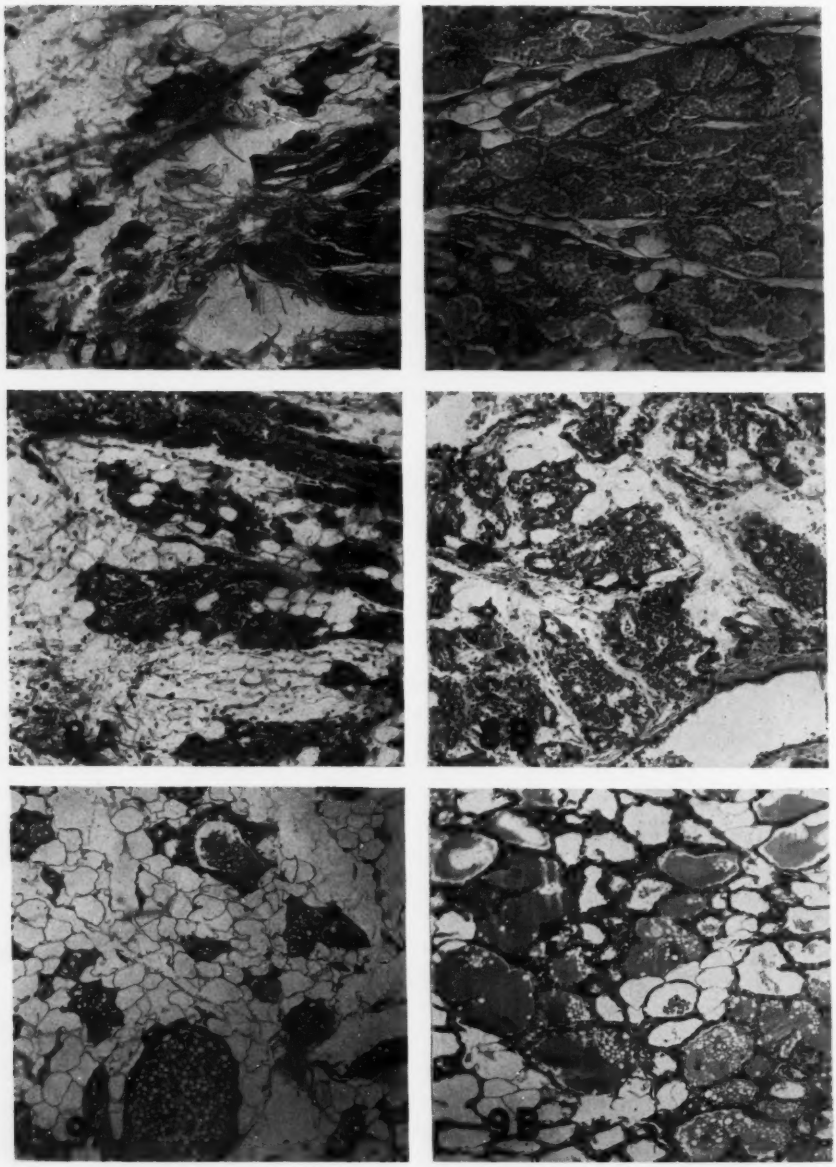

FIG. 7–9. Microphotographs at the same magnification of sections of breasts from rats whose litters had been removed 9 days previously.

FIG. 7A. Section from a control rat injected thrice daily with saline. Note that marked involution of the breasts has taken place leaving only small islands of parenchymal tissue, consisting of cords with only occasional tiny alveoli (Grade = 0).

already described and that they produced at least a partial interruption of the supraoptico-hypophysial tract.

DISCUSSION

The present experiments are the first to our knowledge to demonstrate a failure of milk secretion after hypothalamic lesions. This marked deficit in milk secretion was associated with a failure of milk ejection, indicative of a deficiency of endogenous oxytocin release.

It might be argued that the apparent deficit in milk production found in these rats with lesions was due to an inability of infants to obtain all the milk which was available in the breasts when effective nursing was limited to 1–3 periods a day shortly after an injection of oxytocin. This is not the case since, even after a single shot of oxytocin, fasted litters have been observed to gain at least 10% of their body weight (see section I A). This would mean that, if oxytocin were injected only once a day, the weight gain following oxytocin could equal at least 4.2 mg./g./hr. Such a weight gain was achieved in only one of the litters of the present study (Table 3A, rat 9B), and this mother rat was injected thrice daily with oxytocin. The other litters in the study, many of them also receiving the benefit of three injections of oxytocin, gained from 3.2 down to a minimum of 0.6 mg./g./hr. after oxytocin. The results, therefore, indicate that there is a deficit in milk yield in rats with a deficiency of oxytocin release.

Since limitation of suckling to 8 hours a day has been found to produce a marked reduction in litter weight gain in normal rats (McCann, unpublished), it is conceivable that the observed defect in lactation in rats with lesions might be due to decreased suckling time of litters. This possibility can not be completely ruled out; however, it seems an unlikely one since

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FIG. 7B. Section from a rat injected thrice daily with Pitocin. Note the preservation of alveoli containing secretion (Grade = 4)

FIG. 8A. Section from another control rat injected thrice daily with saline showing essentially the same degree of involution as seen in Figure 7A (Grade = 0).

FIG. 8B. Section from a rat injected with Pitressin thrice daily. Note that, although involution has progressed considerably, alveoli persist which are considerably larger than in the control rats. A large duct containing some secretion can be seen in the lower right hand corner which also distinguishes this section from the controls (Grade = 1).

FIG. 9A. Section from a control rat with a "small" lesion in the supraoptico-hypophysial tract injected thrice daily with saline. Note that, although there is little parenchyma present, there are several fairly large alveoli and two large ducts containing secretion, so that involution has not progressed quite as far as in saline-injected normal rats (Grade = 1)

FIG. 9B. Section from a rat with a "small" lesion in the supraoptico-hypophysial tract injected thrice daily with Pitocin. Many large secretion filled alveoli are present though some alveoli show beginning involution (Grade = 4).

there is no evidence for failure of suckling. Mothers with lesions were observed to suckle their infants as much as normal rats or those with control lesions. In fact, it was notable that in spite of failure to obtain milk, the litters were almost always attached to the teats and that the mothers with lesions retrieved their litters in a normal manner when they were taken from the nest, apparently manifesting normal maternal behavior.

If one grants then that the observed failure of lactation was in fact due to a failure of milk secretion, it would appear probable that this defect was due to the loss of the constellation of pituitary hormones which are required for maintainance of lactation in the rat. That some pituitary hormones were still being secreted by the rats with lesions is suggested by the fact that the deficit in lactational performance in rats with hypothalamic lesions was less than that which followed hypophysectomy in the experiments of Cowie (22).

Since adrenalectomy has an adverse effect on lactational performance in the rat which can be corrected by cortical hormones (23), it might be argued that the defect in lactation following lesions was due to decreased ACTH output by the pituitary. It is known that lesions similar to those reported here can prevent the increased ACTH release which follows acute stress (1–3); however, since complete adrenalectomy produces a much less marked defect in lactation than that reported here, it appears that diminished ACTH secretion alone could hardly account for the present results. Since ovariectomy has little effect on lactational performance in the rat (23), it appears very unlikely that deficits in ovarian secretion secondary to diminished gonadotrophin secretion could explain the results. As growth hormone is capable of increasing milk yields of hypophysectomized rats injected with prolactin (22), it is possible that alterations in growth hormone release may have played a part in the results. Because injections of prolactin are most effective in restoring lactation in the hypophysectomized rat (12), it would appear most likely that the present results were due to a deficiency in the output of pituitary lactogenic hormone (prolactin) and possibly ACTH and growth hormone as well.

The rebound increase in milk yield noted in some of the rats during the first postoperative week is reminiscent of the "normal interphase" of diabetes insipidus. This latter is supposedly due to the release of ADH from the distal ends of the cut fibers of the supraoptico-hypophysial tract (24). Could a similar release of the neurohumor regulating pituitary lactogenic hormone secretion account for the findings in these lactating rats with lesions?

Benson and Cowie (18) have reported that posterior lobectomy abolishes milk ejection in the rat, but that injections of oxytocin permit withdrawal of milk sufficient to sustain litter growth at a rate only slightly below normal. In these experiments, in contrast to those reported here, there was a dissociation between the lack of milk ejection and milk secretion. This

may be accounted for by the possibility that in their experiments there was a secretion of neurohumor from the median eminence into the hypophysial portal vessels. This neurohumor reaching the anterior lobe in high concentration via the portal vessels might be sufficient to stimulate lactogenic hormone release, whereas when it reached the mammary glands after being diluted in the systemic circulation, it would be incapable of inducing milk ejection. Consistent with this idea that some neurohumor (oxytocin) secretion might have still occurred after removal of the neural lobe in their animals is their finding that after remating and parturition no deficiency of milk ejection was present in the succeeding lactation.

Cross and Harris (5) reported a block of milk ejection in rabbits after lesions in the supraoptico-hypophysial tract but found that milk secretion was not greatly affected, again in contradistinction to the present results. Rats apparently differ from rabbits in that in rats the suckling stimuli must continue over most of the 24 hour period to sustain normal milk secretion, whereas Cross and Harris (5) and later Donovan and Van der Werff ten Bosch (25) found normal growth of litters of rabbits allowed to suckle only once daily. It is also possible that the difference between their results and those reported here may have been due to a less complete interruption of the supraoptico-hypophysial tract in their experiments. Recently, Donovan and Van der Werff ten Bosch have similarly reported no interference with milk secretion in rabbits with hypothalamic lesions (25), whereas pituitary stalk section often left milk yield normal but poor in quality. Here, oxytocin was always given to the rabbits with lesions prior to the nursing period of the litter, making it impossible to judge on physiological grounds whether or not a deficiency in the secretion of oxytocin was actually present in their animals.

The results with oxytocin and Pitressin confirm the findings originally reported by Benson and Folley (11) that these materials delay mammary involution. Oxytocin appears to have the major action in this regard though the action of Pitressin is also considerable. It appears likely that the vasopressin molecule may have some intrinsic activity in this regard as analogously vasopressin possesses one fifth the activity of oxytocin in evoking milk ejection (26). It has previously been demonstrated by Bradley and Clarke (27) that injection of oxytocin directly into the ductile system of the breast of rabbits has no stimulatory effect in contrast to the known direct stimulatory action of lactogenic hormone (28). Furthermore, Benson and Folley have shown that oxytocin has no action in delaying involution of the breast in the hypophysectomized rat (11). Consequently, it appears that this action of oxytocin is not exerted locally on the breast and that it requires the presence of the pituitary. Since lactogenic hormone injections produce a similar retardation of involution (9), it is reasonable to suppose that the action of oxytocin is exerted by stimulating a release of prolactin. As the effect of oxytocin persists in the rat with hy-

pothalamic lesions which should block any neural control of lactogenic hormone secretion, it appears likely that oxytocin stimulates the release of lactogenic hormone from the pituitary by a direct action on this gland.

The explanation for the slightly diminished involution of the breast found in the saline-injected rat with lesions is not known. It is conceivable that this represents residual lactogenic hormone secretion in the rat with a lesion. Everett (29) has described persistent luteotrophin secretion in hypophysectomized rats bearing pituitary grafts.

In the rat it appears that pituitary lactogenic hormone and luteotrophin may be identical (30). If this be so, then oxytocin should also stimulate the release of luteotrophin. Indeed, it has already been reported that oxytocin can induce pseudo-pregnancy in the rat (31, 32), and Benson and Folley reported mucification of the vagina in their oxytocin-injected rats (11). Part of the effect of oxytocin in delaying mammary involution may be due to progesterone secretion.

Grosvenor and Turner (33) have reported that oxytocin injection results in a relatively small decrease in pituitary prolactin content when compared to the decrease that follows suckling. They have interpreted this result as being contrary to the thesis that oxytocin stimulates prolactin release. Our results also indicate that the doses of oxytocin injected did not replace the effect of normal suckling stimuli by any means completely. The breasts of oxytocin injected animals were usually not in a fully lactational condition and oxytocin injections did not return lactation to normal in rats with lesions.

As in the case of the effect of vasopressin on ACTH release (1–3), the dose of oxytocin required to produce apparent lactogenic hormone release from the pituitary appears to be much larger than the dose required to produce effects in the periphery, in this case milk ejection. Oxytocin may still play a physiological role in stimulating the pituitary if it reaches the gland in sufficiently high concentration after secretion into the hypophysial portal vessels. Alternatively, it is possible that the effects of oxytocin and vasopressin on the breast occur only at pharmacological doses and that the true neurohumor may be a closely related polypeptide.

Since a deficiency of oxytocin leads to a deficiency of milk secretion presumably mediated by a decrease in lactogenic hormone release, whereas injections of oxytocin appear capable of stimulating lactogenic hormone release by a direct action on the pituitary, it is suggested that oxytocin may be the neurohumor which stimulates the release of lactogenic hormone, or the complex of hormones which maintains lactation. Much more work is necessary to establish this hypothesis.

A surprising incidental finding in the present study was the lack of correlation of oxytocin release as evidenced by milk ejection and ADH release as determined by polydipsia. This could be considered evidence for a view that ADH and oxytocin are synthesized and released by different neurons

of the supraoptico-hypophysial tract. Olivecrona (17) has already suggested that the paraventricular nuclei preferentially secrete oxytocin; however, so far we have no evidence in favor of this particular concept. Rather, it would seem more likely that the cells of both the supraoptic nuclei and paraventricular nuclei secrete either oxytocin or ADH (36).

Acknowledgment

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REFERENCES

1. McCann, S. M.: *Endocrinology* **60:** 664. 1957.
2. Smelik, P. G. and D. de Wied: *Experientia* **14:** 17. 1958.
3. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **19:** 1958.
4. Andersson, B.: *Acta Physiol. Scand.* **23:** 8. 1951.
5. Cross, B. A. and G. W. Harris: *J. Endocrin.* **8:** 148. 1952.
6. Selye, H. and T. McKeown: *Anat. Rec.* **60:** 323. 1934.
7. Reece, R. P. and C. W. Turner: *Proc. Soc. Exper. Biol. and Med.* **35:** 621. 1937.
8. Williams, W. L.: *Yale J. Biol. and Med.* **14:** 201. 1941.
9. Hooker, C. W. and W. L. Williams: *Endocrinology* **28:** 2. 1942.
10. Peterson, W. E.: *Physiol. Rev.* **24:** 340. 1944.
11. Benson, G. K. and S. J. Folley: *J. Endocrin.* **16:** 189. 1957.
12. McCann, S. M., R. Mack and C. Gale: *Fed. Proc.* **17:** 107. 1958.
13. Cowie, A. T. and S. J. Folley: *J. Endocrin.* **5:** 9. 1946.
14. Kluver, H. and E. Barrera: *J. Neuropath. and Exper. Neurol.* **12:** 400. 1953.
15. Gomori, G.: *Am. J. Path.* **17:** 398. 1941.
16. Grosvenor, C. E. and C. W. Turner: *Proc. Soc. Exp. Biol. and Med.* **95:** 131. 1957.
17. Olivecrona, H.: *Acta Physiologica Scand.* **40:** Suppl. 136. 1957.
18. Benson, G. K. and A. T. Cowie: *J. Endocrin.* **14:** 54. 1956.
19. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exp. Biol. and Med.* **87:** 318. 1954.
20. Daniel, P. M. and M. M. L. Prichard: *Quart. J. of Exp. Physiol.* **41:** 215. 1956.
21. Hooker, C. W. and W. L. Williams: *Yale J. Biol. and Med.* **12:** 559. 1940.
22. Cowie, A. T.: *J. Endocrin.* **16:** 135. 1957.
23. Flux, D. S.: *J. Endocrin.* **12:** 57. 1955.
24. O'Connor, W. J.: *Quart. J. Exp. Physiol.* **37:** 1–10. 1952.
25. Donovan, B. T. and J. J. van der Werff ten Bosch: *J. Physiol.* **137:** 410. 1957.
26. van Dyke, H. B., K. Adamsons and S. L. Engel: *Recent Progr. Hormone Research* **11:** 1. 1955.
27. Bradley, T. R. and P. M. Clarke: *J. Endocrin.* **14:** 28. 1956.
28. Lyons, W. R.: *Proc. Soc. Exp. Biol. and Med.* **51:** 308. 1942.
29. Nikitovitch-Winer, M. and J. W. Everett: *Endocrin.* **62:** 522. 1958.
30. Cowie, A. T. and S. J. Folley: *The Hormones* Vol. III. Ed. G. Pincus and K. V. Thimann. Chapter 8, p. 309. 1955.
31. Desclin, L.: *Ann. Endocrin.* Paris **17:** 586. 1956.
32. Stutinsky, F.: *Comptes Rendus Acad. Sci.* Paris **244:** 1537. 1957.
33. Grosvenor, C. E. and C. W. Turner: *Proc. Soc. Exp. Biol. and Med.* **97:** 463. 1958.
34. Andersson, B. and S. M. McCann: *Acta Physiol. Scand.* **35:** 191. 1955.

INHIBITION OF THE DEVELOPMENT OF THE BURSA OF FABRICIUS IN THE EMBRYOS OF THE COMMON FOWL BY 19-NORTESTOSTERONE[1]

ROLAND K. MEYER, M. APPASWAMY RAO
AND RICHARD L. ASPINALL
Department of Zoology, University of Wisconsin, Madison, Wisconsin

ABSTRACT

0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil, when injected into the fertilized eggs of the white leghorn hybrid strain of chickens, "Wisco-white," on the 5th day of incubation, completely inhibits the development of the bursa of Fabricius in both sexes. In the normal chick the bursal primordium appears as a proliferated epithelial mass dorso-caudal to the embryonal cloaca on the 5th day of incubation. By 168 hours of incubation it forms a tubular structure; its terminal end enlarges into a bursal vesicle on the 10th day and becomes lymphoidal by the 15th day. The bursa is fully formed and lymphoidal in 17-day old chick embryos. In 19-nortestosterone-treated embryos the bursal primordium becomes highly vacuolated. Later it remains as a convoluted, short duct without forming the bursal vesicle. Thus the hatched chicks are bursaless. The secondary effects of this hormone on the rectal complex are the stunted growth of the ventral anal lip, increase of musculature in the dorsal lip and an attenuated proctodial canal with very few anal mucoid glands.

IT IS known that the bursa of Fabricius and other lymphatic tissues are considerably reduced by the administration of androgens in young birds (1, 2, 3, 4). Meyer, Aspinall and Buchanan (5) found that when the androgen, 19-nortestosterone, was injected into the egg on the 5th day of incubation and the chicks examined grossly at 19 days or post-hatching, there was a complete absence of the bursa of Fabricius as well as a reduction in size of the thymus and spleen. This discovery provided the stimulus for making a histological study of the organogenesis of the bursa in 19-nortestosterone treated and normal chick embryos. It is the objective of this report to describe the results of this investigation.

MATERIALS AND METHODS

In the chick the bursal primordium appears as a proliferation from the dorso-caudal epithelium of the embryonal cloaca on the 5th day of incubation (6). On this day 0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil was injected at the pointed end of the fertilized egg and the opening in the shell was sealed by paraffin or collodion. Eggs injected with 0.1 ml. of corn oil were incubated with the hormone-treated eggs. The eggs were from a white leghorn hybrid strain of chickens, the "Wisco-white."

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One treated and one control embryo were removed at 8-hour intervals from 120 to 264 hours and thereafter two treated and two control embryos were selected at each 24-hour interval through day twenty-one. Seventy-eight embryos representing 29 stages were made available by this method of selection. One control and one hormone-treated embryo for each day were studied in detail, a total of 58 embryos.

The caudal portions of these embryos were preserved in Bouin's fixative and processed in the usual manner. Sagittal sections of 10 μ thickness were cut and stained in Harris' haematoxylin-eosin.

It was possible to determine the sex of the embryos by visualization of gross characteristics of the gonads only after 11 days of incubation. Thus in 22 treated embryos 10 were males and 12 were females. In 21 control embryos 12 were males and 9 were females.

OBSERVATIONS

Origin and vacuolization of the bursa of Fabricius

a) *128 hours of incubation:* At this stage the bursal primordium in the normal embryos appears as a proliferation of the endodermal epithelium in the dorso-caudal region of the embryonal cloaca (urodeum). Small vacuoles are present in its dorsal aspect (Fig. 1). The origin of the proctodeum is indicated by a caudal depression which is lined by columnar epithelium.

In 19-nortestosterone treated embryos the bursal primordium shows many large vacuoles distributed both dorsally and ventrally (Fig. 2).

b) *168 hours of incubation:* The vacuoles of the dorsal region of the bursal primordium fuse to form a bursal vesicle with a duct communicating with the proctodial canal in the normal chick (Fig. 3). The bursal vesicle and the duct are lined by two to three layers of columnar epithelium, whereas the proctodial canal is lined by cuboidal or flat cells. The vacuoles in the ventral region of the bursal primordium gradually disappear. The proctodial canal is deep and is bounded by dorsal and ventral anal lips which are well developed and are of the same size. The embryonal cloaca extends into the ventral anal lip (Fig. 3).

In 19-nortestosterone treated chicks, the bursal primordium persists as a mass of cells with large ventral vacuoles into which the proctodial canal opens. There is no formation of the bursal vesicle and duct. The proctodial canal is shallow and wide where the proctodeum opens into the ventral aspect of the vacuolated bursal mass. The ventral anal lip is considerably smaller than the dorsal anal lip (Fig. 4).

Growth of the bursal vesicle and plication

In the normal chick, the bursal vesicle, which was narrow in the previous stage, enlarges into a large vesicle after 216 hours of incubation. At 240 hours of incubation the bursal vesicle is large and oval extending dorso-anterior to the embryonal cloaca. Folds arise from the vesicle due to the ingrowth of connective tissue (Fig. 5), and in these folds the cells are loosely arranged and are undergoing mitosis. Some of the epithelial cells are transforming into lymphocytes wherein there is condensation of chromatin. The bursal duct is narrow and long, communicating with the

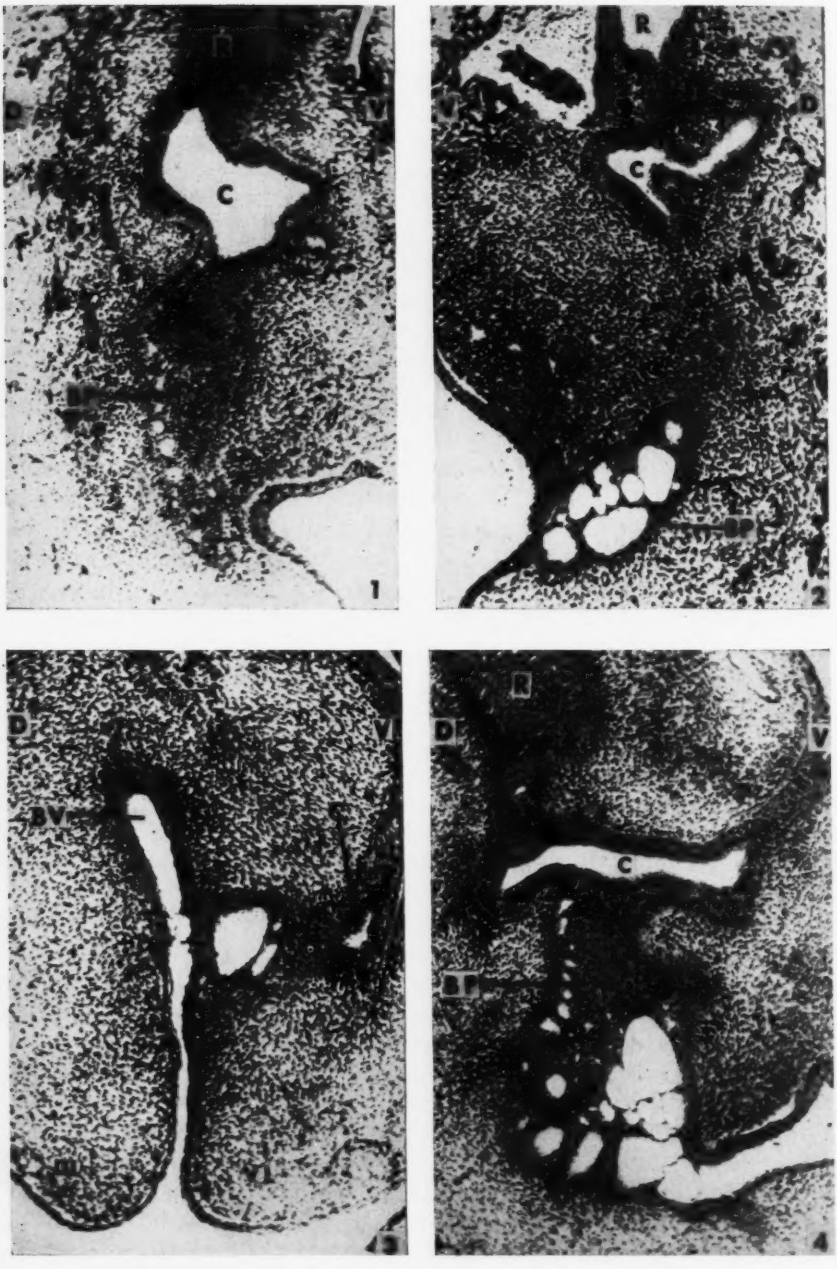

PLATE 1

Sagittal sections of the rectal region of the chick embryos. Fixative: Bouin's solution. Stain: Harris' haematoxylin-eosin.

proctodial canal which is lined by cuboidal cells. The proctodial canal is deep and is bounded by dorsal and ventral anal lips of equal size (Fig. 5).

The bursa in 19-nortestosterone-treated chick embryos is a narrow tube which opens into a narrow, short proctodial canal bounded by dorsal and ventral anal lips of unequal size. The ventral anal lip is short and stumpy (Fig. 7). Surrounding the terminal end of the bursal duct is a diffuse mass of epithelial cells which appears as a dark area (Fig. 7) representing the region of the bursal vesicle. The bursal duct is lined by columnar epithelium which is pseudostratified, especially at the terminal end.

Development of lymphoid follicles in the plicated bursal vesicle

On the 12th day of incubation the entire bursal vesicle in the normal chick is very plicated and solid epithelial buds arise from the folds. In 13 and 14 day old embryos the buds are increased in size and number. During the 15th day these buds are pinched off and many of them are becoming lymphoidal in nature. The bursa in 17 day old normal embryos is large and plicated, with many lymphoidal follicles (Fig. 6). Many eosinophils are found in the vicinity of these follicles. The bursal duct is narrow and communicates with a long and narrow proctodial canal, which is bounded by well developed dorsal and ventral anal lips (Fig. 6). Its ventral wall is thrown into folds while many anal glands are arising as epithelial buds from its dorsal wall (Fig. 6). Both the lips are approximately of the same size and there is slight bend in the dorsal anal lip.

In the 19-nortestosterone-treated embryos the bursal duct is highly convoluted, narrow and surrounded by muscular dorsal anal lip and a narrow, stumpy ventral lip. Unlike normal chicks there are very few small anal glands. These arise from the dorsal wall of the bursal duct (Fig. 8). The rectum, at this stage, has enlarged considerably and is filled with secretions (Fig. 8).

Generalized development of the bursa and proctodeum occurs between

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FIG. 1. 128 hours of incubation in the normal embryo showing the formation of the bursal primordium. Note the vacuoles in its dorsal aspect. ×114.

FIG. 2. 128 hours of incubation, 8 hours after administration of 19-nortestosterone. Note the presence of large vacuoles in the bursal primordium. ×114.

FIG. 3. 168 hours of incubation—Normal. The bursal tube is formed communicating with the proctodial canal. The dorsal and ventral anal lips are of the same size. ×114.

FIG. 4. 168 hours of incubation—Treated. The vacuolated bursal primordium persists. The proctodial canal is wide and shallow, communicating with the ventral aspect of the bursal primordium. ×114.

Abbreviations

B—Bursa of Fabricius.
BD—Bursal duct.
BP—Bursal primordium.
BV—Bursal vesicle.
C—Embryonal cloaca (Urodeum)
D—Dorsal region.
DL—Dorsal anal lip.
R—Rectum.
V—Ventral region.
VL—Ventral anal lip.

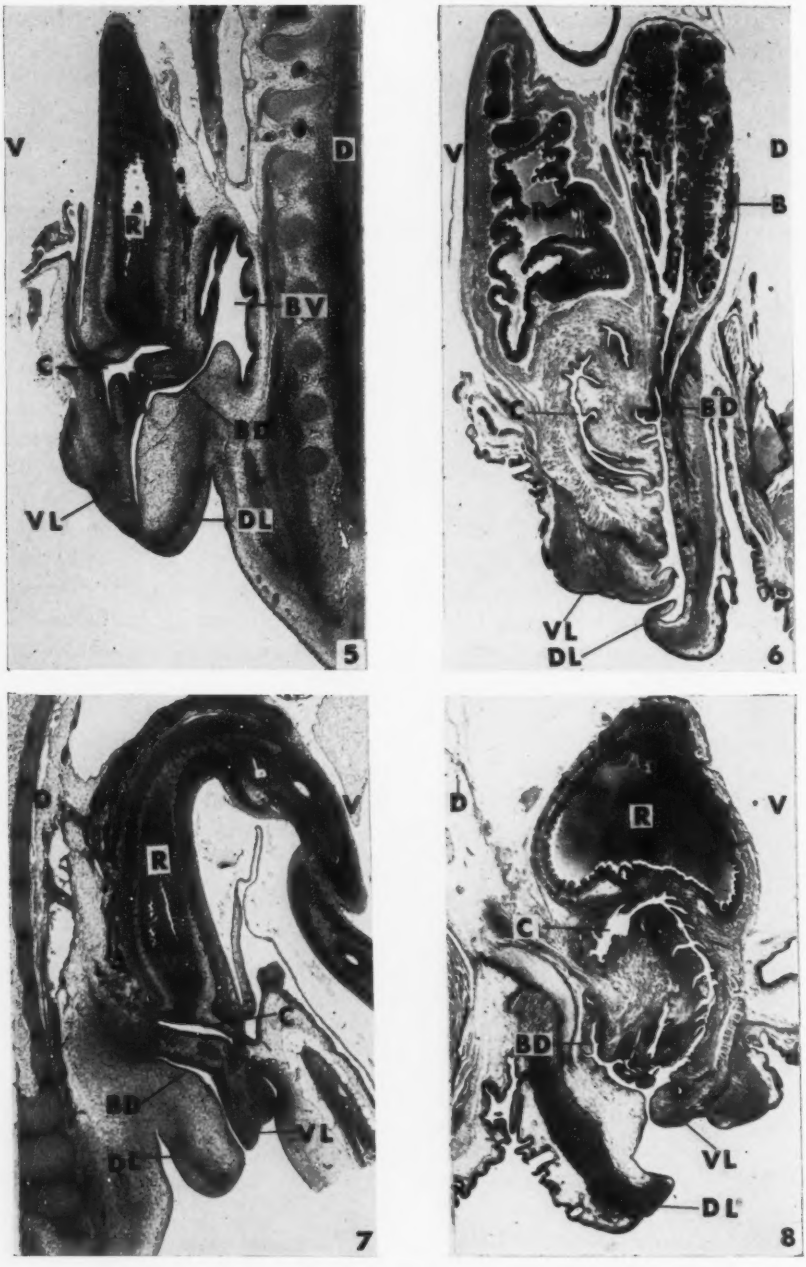

PLATE 2

Sagittal sections of the rectal region of the chick embryos. Fixative: Bouin's solution. Stain: Harris' haematoxylin-eosin.

the 18th and 21st day in normal chicks. The mucoid anal glands also increase in number and size. The bursa, however, is greatly attenuated to a short convoluted duct in 19-nortestosterone-treated chicks. The proctodial canal is short and narrow, bounded by a muscular dorsal anal lip and a short, stumpy ventral anal lip. Very few mucoid anal glands develop. The rectum is large and thin-walled.

DISCUSSION

Effect of 19-nortestosterone in suppressing the development of the bursa of Fabricius

Whether male gonadal hormones are lympholytic or inhibit the formation of new lymphocytes is still debated. Since there is accelerated destruction of these cells in intact animals as compared to gonadectomized animals, Dougherty (7) thought that androgens were lympholytic. Brauchhardt (8) noted that testosterone propionate inhibited mitosis of the epithelial cells of the intestine of guinea pigs and causes considerable reduction in lymph node weight. Agrel and Persson (9) indicated that testosterone propionate inhibited the formation of desoxypentose nucleic acid and correspondingly blocked cellular cleavage.

Histological changes of the bursa in treated embryos show that in the initial stages there is vacuolization of the bursal primordium (Figs. 2 and 4), but macrophages are not present, nor is there any degeneration of epithelial cells in that area as seen in the involuting lymphoid tissues. In the later stages the bursa remains as a convoluted, short duct and the bursal vesicle does not develop (Figs. 7 and 8). It is interesting to note that irrespective of the sex the bursal development is inhibited by 19-nortestosterone. This may be attributed to the suppression of the mitotic activity of the cells in the terminal region of the bursal duct, thus confirming the views held by Brauchhardt (8) for the thymus and other lymphoid tissues.

In another series of experiments, the results of which will be described in another report, it was found that in the newly hatched chicks treated with 19-nortestosterone on 5th day of incubation, the thymus and spleen,

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FIG. 5. 240 hours of incubation—Normal. The bursal vesicle is fully formed within which folds are appearing. Dorsal and ventral anal lips are of the same size. ×21.

FIG. 6. 17 days of incubation—Normal. The bursa is fully formed with many lymphoid follicles. The proctodial canal is long, bounded by well developed dorsal and ventral anal lips. Note the presence of many anal glands along the dorsal wall of the proctodial canal. ×13.

FIG. 7. 240 hours of incubation—Treated. The bursal vesicle is absent and that region is represented by a mass of epithelial cells stained dark at the terminal end of the bursal duct. The ventral anal lip is short and stumpy. ×21.

FIG. 8. 17 days of incubation—Treated. The bursa is absent, and the bursal duct is greatly reduced. The proctodial canal is short and narrow. A few small anal glands are present in the basal region of the bursal duct. Note the well developed musculature in the dorsal lip and the short, stumpy ventral lip. ×13.

although present, were reduced 30 to 50% when compared to those of non-treated chicks.

Although the present experiment was designed to suppress the development of the bursa in the chick, there were some secondary effects of this hormone on the rectal complex. Both dorsal and ventral anal lips were long and well developed in the normal chicks and the proctodial canal was long and lined by many large mucoid glands (Figs. 5 and 6). In 19-nortestosterone treated chicks there was, however, unequal growth of these lips; the ventral anal lip was short and stumpy but the dorsal lip was highly muscular. The latter may have been due to the myotrophic effect of this hormone and the former due to the delay in the growth of the posterior extension of the cloaca (Figs. 4, 7 and 8). This delay in the growth of the posterior extension of the embryonal cloaca may be due to the retarded growth and to the presence of the vacuolated bursal primordium in the posterior region of the cloaca (Fig. 4). Consequently, the proctodial canal was narrow and short with very few anal mucoid glands. It is suggested that this caused the feces to accumulate in the cloaca, thus plugging the vent. The plugging may be due in part to the short ventral anal lip and muscular dorsal lip and to the insufficient production of mucous secretion which acts as a lubricant in the normal chick. These effects were seen in the pheasant as well as in the chickens (10).

Significance of this experiment

The function of the bursa of Fabricius is still debated as bursectomy has no influence either on the somatic growth or on the gonads except for a slight increase in the size of the ovary (11, 12, 13, 14, and 3). Riddle and Krizenecky (11) could not observe any appreciable effect after bursectomy and thymectomy in body growth and gonadal activity in pigeons. These lymphoid tissues are so widely distributed that bursectomy and thymectomy are without appreciable effect. Gyllensten (15) and Gyllensten and Ringertz (16) have shown that partial thymectomy in guinea pigs causes hyperplasia of other lymphoid tissues. Recently, Metcalf and Buffett (17) have shown in normal, thymectomized and adrenalectomized mice a lymphocytosis response after the administration of thymic extract which, they believe, is caused by some "lymphocytosis stimulating factor" present in the thymus and not in the lymph nodes. Comsa (18) and Comsa and Leroux (19), however, believe that in guinea pigs the lymphocytosis caused by thymus extract is mediated through the adrenal in which there is hyperplasia of the medulla and a considerable reduction in the cortical region. Glick (3) indicates that bursectomy reduces the production of antibodies. Therefore, a complete suppression of the bursa with sufficient atrophy of other lymphoid tissues, especially thymus, by 19-nortestosterone may open further avenues for elucidating the above mentioned problems, especially the function of the bursa and other lymphoid tissues in general.

SUMMARY

0.63 mg. of 19-nortestosterone in 0.1 ml. of corn oil, when injected on the 5th day of incubation into the eggs of Wisco-white chickens, completely inhibits the development of the bursa of Fabricius. The hatched chicks are, therefore, bursaless.

In the normal chick the bursal primordium appears as a proliferated epithelial mass in the dorso-caudal region of the embryonal cloaca on the 5th day of incubation. By 168 hours of incubation, it forms a tubular structure communicating with the proctodial canal. Its terminal end enlarges into a vesicle after 216 hours. On the 10th day the bursal vesicle becomes plicated; on the 12th day epithelial buds are given off which become lymphoidal in nature by the 15th day. Thus, the bursa is fully formed and lymphoidal in 17 day old embryos.

There is vacuolization of the bursal primordium in early stages in 19-nortestosterone-treated embryos. It remains as a convoluted short bursal duct without a bursal vesicle. It has been suggested that this may be due to the suppression of mitosis in the bursal primordium.

The secondary effects of 19-nortestosterone on the rectal complex are the stunted growth of the ventral anal lip, increase of musculature in the dorsal lip and attenuation of the proctodial canal with few mucoid glands. These effects are correlated with the accumulation of feces in the cloaca.

Acknowledgment

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REFERENCES

1. Selye, H.: *J. Morph.* **73**: 401. 1943.
2. Kirkpatrick, C. M. and F. N. Andrews: *Endocrinology* **34**: 340. 1944.
3. Glick, B.: *Doctoral dissertation, Ohio State University.* 1955.
4. Höhn, E. O.: *Canadian J. Biochem. & Physiol.* **34**: 90. 1956.
5. Meyer, R. K., R. L. Aspinall and K. E. Buchanan: Unpublished Observations.
6. Jolly, J.: *Arch, D'anat. Micro.* **16**: 365. 1914.
7. Dougherty, T. F.: *Physiol. Rev.* **32**: 379. 1952.
8. Brauchhardt, G.: *Zeitschrift für Vitamin-, Hormon- und Fermentforschung.* **5**: 373. 1953.
9. Agrel, I. and H. Persson.: *Nature* **178**: 1398. 1956.
10. Rao, M. A., R. L. Aspinall and K. E. Buchanan.: *Anat. Rec.* **130**: 459 (Abstract). 1958.
11. Riddle, O. and J. Krizenecky: *Am. J. Physiol.* **97**: 343. 1931.
12. Taibel, A. M.: *Riv. di. Biol.* **24**: 364. 1938.
13. Taibel, A. M.: *Riv. di. Biol.* **39**: 164. 1947.
14. Hammond, J. C. and H. R. Bird: *Poul. Sci.* **21**: 116. 1942.
15. Gyllensten, L.: *Acta Anat.* **22**: 82. 1954.
16. Gyllensten, L. and N. R. Rigertz: *Acta Path. et Microbiol. Scand.* **35**: 309. 1954.
17. Metcalf, D. and R. F. Buffett: *Proc. Soc. Exp. Biol. & Med.* **95**: 576. 1957.
18. Comsa, J.: *Acta Endoc.* **19**: 406. 1955.
19. Comsa, J. and H. Leroux: *J. Endocrin.* **13**: 7. 1956.

THE DISTRIBUTION OF RADIOACTIVITY IN NON-EXCRETORY ORGANS OF THE MALE RAT AFTER INJECTION OF TESTOSTERONE-4-C^{14} [1,2,3]

DAVID S. GREER[4]

The Department of Medicine, The University of Chicago, Chicago, Illinois

ABSTRACT

After the subcutaneous injection of physiologic amounts (about 0.1 mg.) of testosterone-4-C^{14} of high specific activity, significant concentrations of radioactivity were found in several non-excretory tissues of pubescent rats.

The concentrations of radioactivity localized in ventral prostates and other accessory sex glands averaged about two and a half times as great as that in muscle.

The average prostatic concentration of radioactivity in recently castrated animals was not significantly different from that detected in intact pubescent rats, but the levels were more variable from animal to animal in the castrates.

The localization of radioactivity after testosterone-4-C^{14} administration was not influenced by the presence of biliary fistulae.

It is suggested that the radioactivity detected in the tissues probably represented androgen or a derivative.

ENDOCRINE regulation of metabolic processes involves the production, secretion, transport, and utilization of minute amounts of hormonal material. *In vivo,* at the target organ level, the quantity of hormonal substance required to produce profound metabolic and anatomic changes is often so small that it is not detectable by even the most sensitive chemical procedures. Isotope techniques, however, permit detection of such minute quantities of material that physiologic amounts of isotopically labelled hormones might be determinable in tissues.

Previous investigators have been unable to find significant amounts of radioactivity in the accessory sex organs of male animals after the administration of radioactive labelled androgen. Barry, Eidinoff, Dobriner and Gallagher (1) administered testosterone-4-C^{14} intraperitoneally in propylene glycol to rats and mice; but their hormone preparation was of low specific activity (each animal received only 50,000–175,000 disintegrations

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[2] Testosterone-4-C^{14} obtained from Tracerlab, Inc., Waltham, Mass.

[3] Presented at the November, 1957 meeting of the Central Society for Clinical Research and published in the abstracts of that meeting.

[4] Present address: Truesdale Hospital, Fall River, Kan.

per minute) and significant concentrations of radioactivity were not detected in the sex organs of their animals. Holmes, (2) although primarily interested in the transport of testosterone by plasma proteins, also failed to find significant radioactivity in the sex organs of mature male mice after the administration of testosterone-4-C^{14}.

In the studies to be reported here testosterone-4-C^{14} of high specific activity was dissolved in sesame oil and administered subcutaneously to castrated and intact pubescent male rats in an effort to demonstrate the localization of the isotope in responsive tissues.

METHODS

In all experiments an attempt was made to duplicate physiologic conditions as closely as possible. The dose of testosterone approximated the estimated requirement of 0.1 mg. per day (3) and the steroid was dissolved in sesame oil and injected subcutaneously with the expectation that slow absorption from a depot would simulate the probable continuous delivery of androgen from the testis. Initially, recently castrated pubescent animals were used in order to provide optimal conditions for demonstrating

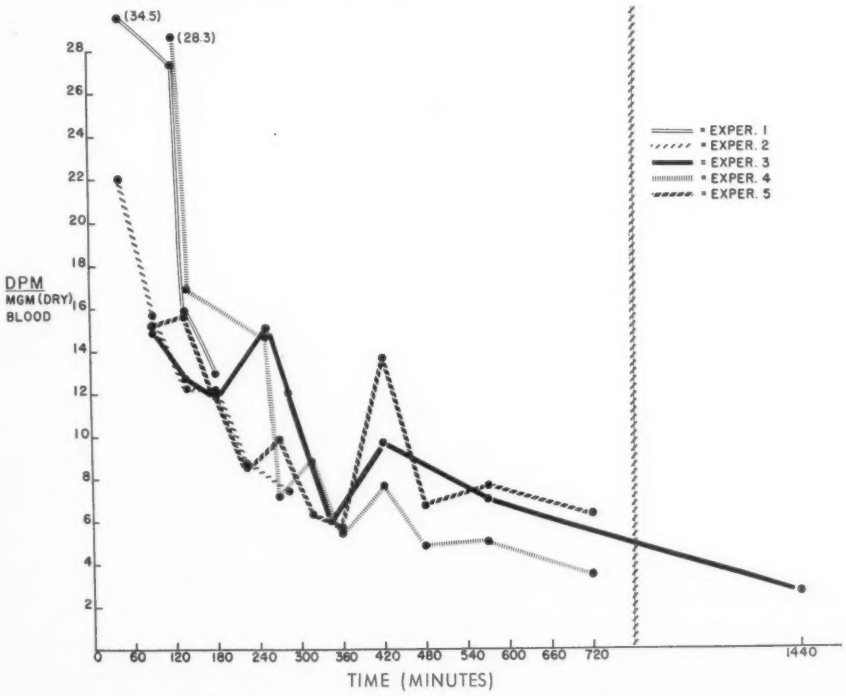

FIG. 1. Radioactivity (disintegrations per minute per mgm. dry weight) in the blood of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the blood of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

androgen localization in accessory sex glands. Hooker (4) showed that pubertal sex accessory tissue is more sensitive to androgen than pre-pubertal or mature glands, and it was felt that this enhanced sensitivity might be associated with an increased avidity of these tissues for testosterone. Castration, by removing the source of endogenous testosterone, would produce a state of androgen need in the partially developed accessory sex tissues.

Testosterone-4-C^{14} with a specific activity of approximately 12 microcuries per milligram was dissolved in sesame oil so that the final stock solution contained 6.5 micro-

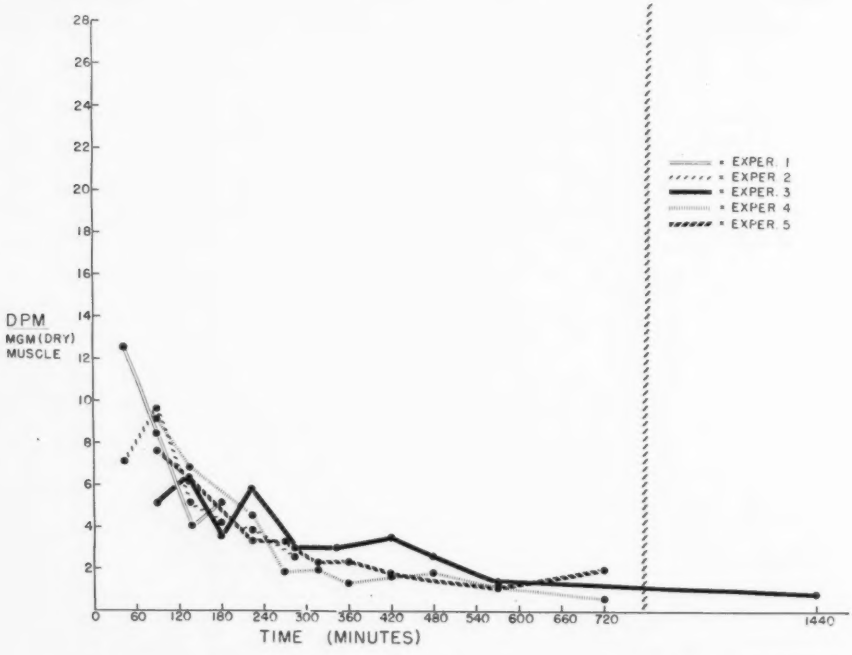

FIG. 2. Radioactivity (disintegrations per minute per mgm. dry weight) in the gastrocnemius of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the gastrocnemius of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

curies per ml. The experiments in which animals were sacrified at various post-injection times (Figs. 1, 2, 3) were done with 45 day old Sprague-Dawley rats which had been castrated three days previously. Each animal was given a 0.2 ml. subcutaneous injection of the stock solution in the dorsal neck region. Care was exercised to avoid leakage of the oily solution from the injection site, and the injected area was vigorously massaged to insure even diffusion of the solution throughout the subcutaneous tissues. The animals were fed Purina chow for several days prior to the experimental period, but were given no food or water after the testosterone injection.

At the time of sacrifice (with ether), a heart blood sample was obtained and then a large bore needle was inserted into the aorta of each animal through the beating left ventricle. The inferior vena cava was cut just above the diaphragm, and the animal

was perfused with 100 ml. of normal saline at a pressure of 4–6 feet of water. The sex organs, which invariably appeared bloodless, were then excised under a dissecting lens, blotted, and homogenized in 2.5 ml. of water using glass tissue grinders. Samples of gastrocnemius were homogenized in a similar fashion. Aliquots of each homogenate and of diluted blood specimens (2 ml.) were transferred to weighed copper planchets and evaporated to dryness. The planchets were then reweighed and radioactivity was measured in windowless gas-flow counters. Corrections were made for self-absorption.

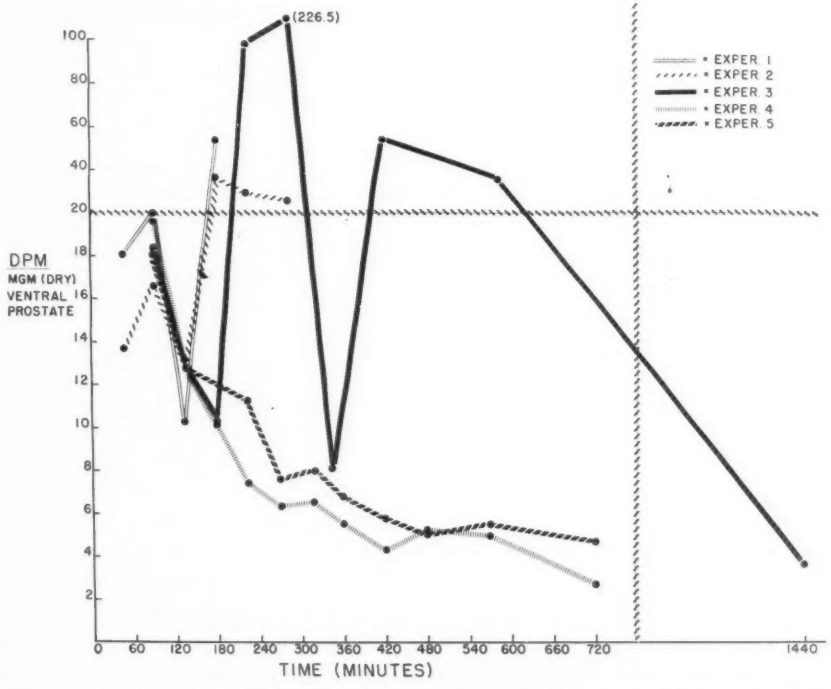

FIG. 3. Radioactivity (disintegrations per minute per mgm. dry weight) in the ventral prostate of 45-day old Sprague-Dawley rats which had been given 1.3 microcuries of testosterone-4-C^{14}. Each dot represents the radioactivity in the ventral prostate of an animal killed at the time indicated after administration of the isotope. Lines connect the values secured on one experimental day.

All values were expressed as disintegrations per minute per milligram dry weight of tissue.

In a second series of experiments in which all animals were sacrificed 300 minutes post-injection, 52-day old male Sprague-Dawley rats weighing approximately 200 gm. were used. These animals were fed a hormone-free diet containing multivitamins, and adequate salt mix, casein, alphocel, dextrin, Mazola oil, oleum percomorpheum and water for several days prior to the experimental period (5). Some of these animals were castrated two days prior to the injection of testosterone-4-C^{14}. Others from the same lot were used intact. Each of these animals received 0.01 ml. of the stock solution per 10 gm. body weight. None of these animals was perfused. The muscle used for comparison was

tongue. Tissues were blotted and weighed before homogenization on a Roller-Smith torsion balance. All other procedures used were the same as those noted above except that all values were expressed as disintegrations per minute per 10 mg. wet weight of tissue.

RESULTS

Significant amounts of radioactivity were found in the blood, muscle, accessory sex glands, testis, adrenals, and intestinal walls of pubescent male rats after the administration of C-14 labelled testosterone. In Figure 1 the concentration of radioactivity in the blood of 3-day castrated 45-day old rats is plotted against time. Although there were some irregularities, it can be readily seen that the concentration of isotope dropped rapidly during the first four hours and then more slowly, the biological decay rate approaching an exponential function. Radioactivity was detected 24 hours after administration in one animal. The radioactivity found in gastrocnemius muscle followed a parallel course but was lower throughout (Fig. 2). The isotope concentration in the ventral prostates of these animals also followed the same general course (Fig. 3) but the level was always higher than that observed in the muscle and often greater than that in the blood of the same animals. Since perfusion removed very little of the radioactivity, it may be presumed that the isotope is muscle and prostate was located in extravascular spaces probably bound to the cells.

In occasional animals very high concentrations of radioactivity were found in the prostate. These unexpected results could not be explained by technical or dietary factors, both of which were exhaustively searched for in other experiments. It may be that these exceptional values were related to the age of the rats, for they were never noted in 52-day old animals.

For the purpose of developing a standard procedure which could be utilized for physiologic comparisons, a 300 minute interval following administration of testosterone-4-C^{14} was chosen as the time for sacrificing rats. This time was selected because at 300 minutes the rate of decline in the concentration of radioactivity in the tissues was becoming less rapid, but the isotope concentration was still sufficiently high to permit relatively short counting times. Fifty-two day old rats were used because, as previously mentioned, the uptake of C^{14} by the ventral prostate seemed to vary less from animal to animal at this age.

A new device for presenting isotope concentration data was evolved, the ventral prostate/muscle ratio. This expression tends to cancel the effect of variations in circulating levels of radioactivity and thus makes results obtained at different blood levels more comparable (see below). The tongue, consisting largely of muscle which is not androgen dependent, is then a "baseline" tissue, and the ratio becomes an index of the avidity of the prostate for androgen-derived radioactivity.

Under these conditions the special isotope localizing capability of ventral prostate was clearly brought out (Table 1). With but two excep-

tions the concentration of isotope in blood was exceeded by that in the ventral prostate. In both the castrated and intact animals the radioactivity in the prostates was with one exception substantially greater than that in tongue muscle. In castrates the prostatic radioactivity averaged 2.87 times as much as muscle; in intact rats the prostate concentrated 2.46 times as much (P of both groups less than 0.001). There was no significant difference between the average ratios in castrate and intact rats, but the values in individual animals were more variable in the castrates.

TABLE 1. THE CONCENTRATION OF RADIOACTIVITY (DPM/10 MG. WET WEIGHT) IN THE BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF 52-DAY-OLD RATS SACRIFICED 300 MINUTES AFTER INJECTION OF TESTOSTERONE-4-C^{14}

Castrate animals				Intact animals			
Blood	Ventral prostate	Muscle	VP/M	Blood	Ventral prostate	Muscle	VP/M
7.1	13.1	3.0	4.37	3.2	12.4	3.9	3.18
12.2	21.2	8.1	2.62	3.7	8.8	4.1	2.12
9.1	14.5	6.0	2.42	3.9	10.1	3.3	3.06
4.9	16.1	2.9	5.55	9.8	14.1	10.7	1.32
5.7	17.2	4.8	3.58	3.7	8.7	4.3	2.02
7.5	17.1	6.0	2.85	6.0	10.4	4.8	2.17
10.1	12.4	7.6	1.63	6.6	14.4	5.6	2.52
14.3	16.0	6.0	2.67	6.6	15.0	5.6	2.68
13.4	18.3	6.4	2.86	5.8	13.0	4.6	2.83
4.8	11.9	4.6	2.59	3.0	11.9	4.3	2.77
5.6	14.7	4.8	3.06	6.2	12.6	5.2	2.42
14.0	9.3	10.2	.91				
7.5	12.1	4.6	2.63				
9.5	9.1	5.9	1.54				
13.7	14.1	8.7	1.62				
6.5	14.4	4.4	3.27				
6.0	16.0	5.5	2.91				
7.2	16.8	4.8	3.50				
11.3	16.6	4.5	3.69				
8.9	16.8	4.8	3.50				
6.4	16.2	6.4	2.53				
4.7	14.8	4.7	3.15				
5.1	9.9	3.9	2.54				
Mean 8.5	14.7	5.6	2.87	5.3	11.9	5.1	2.46

The influence of blood levels of the isotope was demonstrated by administering several doses of testosterone-4-C^{14} (Table 2). It can be seen that the level of isotope in blood rose progressively as the dosage of labelled testosterone was increased, and the concentration in the tongue tended to follow that of blood rather closely. The concentration of isotope in ventral prostate, however, did not follow that of blood consistently but was higher than that in blood or muscle at all three dosages.

It would be premature to state at this time that the radioactivity found represented testosterone or a physiologically active androgen derived from it. Preliminary experiments have, however, suggested that the isotope content of the tissues has some direct relationship to the administered androgen. The major route of excretion of radioactivity derived from tes-

TABLE 2. EFFECT OF VARYING THE DOSE OF TESTOSTERONE-4-C^{14} ON THE CONCENTRATION OF RADIOACTIVITY (DPM/10 MG. WET WEIGHT) IN BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF 52-DAY-OLD RATS CASTRATED 48 HOURS PRIOR TO INJECTION AND SACRIFICED 300 MINUTES POST-INJECTION

Dose	Blood	Muscle	Ventral prostate	VP/M
.1 cc.	2.2	1.7	5.7	3.35
.2	3.5	2.8	8.0	2.85
.3	4.1	3.7	6.5	1.75
.1	1.7	1.4	5.3	3.78
.2	2.3	2.2	5.7	2.59
.3	4.4	4.0	10.1	2.52
.1	1.2	0.6*	5.1	8.50
.2	2.7	2.3	7.1	3.09
.3	3.3	3.2	5.8	1.81

* Concentration too low for significant count.

tosterone in the rat is via the liver (1). It is therefore of significance that common bile duct cannulation and consequent elimination of liver-excreted radioactivity did not change the absolute concentration of radioactivity in the ventral prostate or the ventral prostate tongue ratio (Table 3). The tissue radioactivity measured thus could not represent hepatic or intestinal degradation products of testosterone-4-C^{14} which had been reabsorbed from the gastroinestinal tract.

The radioactivity in the seminal vesicles was of the same order of magnitude as that found in ventral prostates after the administration of testosterone-4-C^{14}, but the concentrations were more variable from animal to animal. In addition to muscle, salivary glands, adrenals, testes and the colons of bile cannulated animals were sampled, but in none of these tissues was there as high a concentration of the isotope as in the accessory sex glands. The higher levels of androgen derived radioactivity thus seemed to be confined to androgen dependent tissues.

DISCUSSION

The fact that radioactivity was localized in non-excretory tissues after testosterone-4-C^{14} administration in these studies in contrast with the failure to demonstrate such localization by Barry, *et al.* (1) can probably be explained on the basis of the high specific activity of this testosterone. With

TABLE 3. THE CONCENTRATION OF RADIOACTIVITY (DISINTEGRATIONS PER MINUTE PER 10 MGM. WET WEIGHT) IN THE BLOOD, VENTRAL PROSTATES AND MUSCLE (TONGUE) OF RATS WITH CANNULATED BILE DUCTS

	Blood	Prostate	Muscle	VP/M
	—	24.9	4.9	5.08
	12.3	15.7	11.0	1.43
	5.3	13.8	6.0	2.30
	9.4	12.3	5.1	2.41
	1.5	4.7	2.6	1.80
Mean	7.12	14.3	5.9	2.60

the product available, it was possible to inject $2.5–3.0 \times 10^6$ D.P.M. and yet keep the dose of testosterone within the physiologic range of about 0.1 mg. per animal.

Despite the large amount of radioactivity injected tissue levels five hours after injection were in the range of 10–20 D.P.M. per 10 mg. wet weight of tissue. Such a yield may seem insignificant, but dilution in the animal is immense. The ventral prostate comprises less than 0.1% of the total volume of distribution in 52-day old rats. Absorption from sesame oil is slow, and excretion of radioactivity rapid; blood concentrations therefore never achieve very high levels. At five hours post-injection it seems likely, from our experience and that of previous workers (1, 2), that most of the injected radioactivity is in excretory organs. Precise calculation is impossible but these considerations have led us to conclude that yields of radioactivity of the order of magnitude reported in this study were to be expected.

The fact that muscle and other tissues which are not frankly androgen dependent attracted and held some of the radioactive isotope calls for some comment. Testosterone promotes protein anabolism generally (6, 7), and this process might well require the localization in muscle of the androgen or a derivative in a manner similar to that noted in accessory sex tissues. The close correlation between the blood levels of radioactivity and the concentration in muscle suggests that the muscle localization may be a relatively passive phenomenon. The substantially greater concentration in accessory sex glands would accordingly point to the existence of a more active localizing process in androgen-dependent organs. However, it is also possible that this difference may be due to the relative rates at which the tissues involved metabolize the isotope containing compound to another which does not remain in situ. Thus a more rapid dissipation of the androgen or its derivative in muscle would lead to a lower tissue level of isotope than that found in the ventral prostate.

These results do not prove that testosterone is localized in the process of regulating cellular activities. Nevertheless, the fact that those organs which are most dependent upon androgen for their growth and function are the ones which maintain the higher concentrations of isotope after the administration of testosterone-4-C^{14} is suggestive. The identification of the isotope-bearing compound in the tissues is obviously of prime importance, but the significance of these findings would be much the same if the radioactivity represented a metabolite or metabolites of testosterone. The regulation of such a suggested localizing mechanism as well as the controlling of the rate of dissipation of androgen by tissues could be important aspects in the endocrine control of tissue metabolism.

Acknowledgment

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REFERENCES

1. Barry, M. C., M. L. Eidenoff, K. Dobriner and T. F. Gallagher: *Endocrinology* **50**: 587. 1952.
2. Holmes, W. N.: *Acta Endocrinologica* **23**: 89. 1956.
3. Moore, C. R. and D. Price: *Anat. Rec.* **71**: 59. 1938.
4. Hooker, C. W.: *Endocrinology* **30**: 77. 1942.
5. Wissler, R. W., R. L. Woolridge, C. H. Steffee and P. R. Cannon: *J. Immunology* **56**: 267. 1946.
6. Kochakian, C. D.: *Endocrinology* **21**: 750. 1937.
7. Kenyon, A. T., I. Sandiford, A. H. Bryan, K. Knowlton and F. C. Koch: *Endocrinology* **23**: 135. 1938.

WATER AND ELECTROLYTE CONTENT OF THE UTERUS OF THE INTACT AND ADRENALECTOMIZED RAT TREATED WITH RELAXIN AND VARIOUS STEROID HORMONES[1]

DAVID M. BRENNAN[2] AND M. X. ZARROW

Department of Biological Sciences, Purdue University, Lafayette, Indiana

ABSTRACT

The effect of relaxin[3] administration on the water and electrolyte content of the uterus of the intact and adrenalectomized, immature rat was determined. Uterine water response to the simultaneous administration of estradiol and relaxin was also measured. Relaxin (10 G.P.U.) caused a uterine water increase from a control value of 80.2% to 84.2%. Adrenalectomy caused a 4 to 6-fold increase in sensitivity to relaxin and an increase in the magnitude of the response. Simultaneous administration of minimally effective doses of relaxin and estradiol prevented the uterine water increase while intermediate doses resulted in an additive effect. Progesterone (2 mg.) partially inhibited the action of 10 G.P.U. of relaxin while 1.25 mg. of cortisone acetate completely inhibited the action of 10 G.P.U. of relaxin. The concentration of sodium underwent a slight increase at six hours following relaxin administration but at eighteen had fallen significantly below the normal level. Potassium concentration underwent a slight decrease at six hours, but at eighteen hours had risen above the control level.

IT HAS been known for a number of years that cyclic fluctuations in the water content of the uterus are under hormonal control (1), and that estrogen administration to immature rats causes an increased uterine water content, comparable to that observed in late proestrus (2). It has been shown recently that relaxin administration to rats produces an increase in uterine water content, when given alone (3), or following pretreatment with estrogen (4).

The present report is concerned with the relationship of relaxin to the steroid hormones in its action on the water and electrolyte content of the uterus in the intact and adrenalectomized rat. Specific attention was paid to the influence of estradiol, progesterone, and cortisone on the action of

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[2] Present address: Endocrine Research Department, Squibb Institute for Medical Research, New Brunswick, New Jersey.

[3] Relaxin was supplied under the trade name of Releasin (Lot No. 004093) through the courtesy of Dr. Robert L. Kroc, Warner-Chilcott Laboratories, Morris Plains, New Jersey.

relaxin. Sodium and potassium concentrations in the uterus were determined at various intervals following the administration of relaxin, in an effort to elucidate the mechanism by which uterine imbibition occurs.

MATERIALS AND METHODS

Immature rats of the Holtzman or Purdue strain were used throughout these studies. The animals were weaned at 21 days of age, and placed upon a liquid diet (10% glucose in the drinking water) for three days. This was found necessary in order to insure a consistent uterine water content of approximately 80% in the untreated animals. Earlier studies had revealed abnormally high concentrations of water in normal uteri, which could be explained by the presence of estrogen in the food (5). In determining the role of the adrenal cortex on this phenomenon, animals were bilaterally adrenalectomized on the day of weaning, and placed on a liquid diet, (10% glucose and 1% sodium chloride in the drinking water). In addition, each animal received a single subcutaneous injection of 500 μg. desoxycorticosterone acetate immediately following adrenalectomy.

Estradiol was dissolved in sesame oil and injected in doses ranging from 0.005 μg. to 0.1 μg. Relaxin solutions were prepared from a stock solution of Releasin,[3] and administered in doses ranging from 0.05 G.P.U. to 100 G.P.U. All injections were made subcutaneously, and each injection was given in a volume of 0.1 ml. In previous experiments (2, 3), it was demonstrated that maximum uterine water concentration following a single injection of either 0.1 μg. estradiol or 50 G.P.U. relaxin occurs six hours after administration. Accordingly all treated animals were killed six hours following injection.

The animals were killed by ether, weighed, and the uterus quickly removed from the body cavity. The uterus was dissected free of fat, and a longitudinal incision made in the antimesometrial surface, extending from the ovarian end to the cervical junction, in order to allow the escape of lumenal water. The uterus was then gently rolled on bibulous paper to remove water adhering to the outer surface, and weighed to the nearest 0.1 mg. on a torsion balance. The uteri were dried in a vacuum oven at 50° C, weighed again, and the water content calculated.

Dried uterine tissue was wet ashed in a 2 ml. mixture containing five parts concentrated nitric acid to one part perchloric acid. This mixture was then dry ashed at 550° C for 12 hours. The ashed residue was brought to 10 ml. volume with triple distilled water, and analyzed for sodium and potassium content in the flame photometer. The "direct intensity" method was used for determination of both ions, since interfering ions were absent in the concentrations used.

RESULTS

The lowest dose of relaxin which increased significantly the per cent of water in the uterus of the intact rat was 1 G.P.U. (Fig. 1). This dose of relaxin increased the per cent uterine water from a control value of 80.2% to a value of 81.4%. Although an effect was obtained following the administration of 0.1 G.P.U. statistical analysis by the Newman-Keuls sequential range test demonstrated no significant difference at the 1% level. A maximum response (84.2% water) was obtained following the injection of 10 G.P.U. relaxin. Further increase in the dosage of relaxin to 50 and 100 G.P.U. failed to elicit a significantly greater response.

The administration of progesterone partially inhibited the increase in

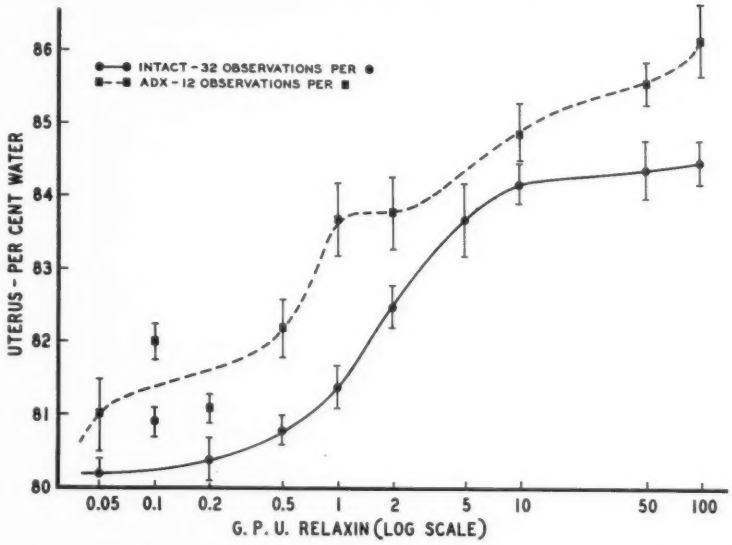

FIG. 1. Increase in the per cent uterine water in the immature rat treated with relaxin.

●—intact rat
■—adrenalectomized rat

uterine water content observed following administration of relaxin alone (Table 1). Treatment with 10 G.P.U. of relaxin and 2 mg. of progesterone increased the uterine water to 81.7% as compared with 84.2% for relaxin alone. Lower dosages of progesterone with the relaxin held constant at 10 G.P.U. produced a correspondingly smaller degree of inhibition. Doses of 0.25 and 0.5 mg. progesterone failed to induce any inhibition of the action of 10 G.P.U. of relaxin on the uterine water content, while 1 mg. of progesterone caused a 41% inhibition and 2 mg. progesterone caused a 61% inhibition.

Adrenalectomy increased the uterine water response to relaxin. Although

TABLE 1. UTERINE WATER CONTENT OF THE 24-DAY-OLD RAT AT 6 HOURS FOLLOWING THE SIMULTANEOUS ADMINISTRATION OF RELAXIN AND PROGESTERONE

No. of animals	Relaxin G.P.U.	Progesterone, mg.	Uterine water— %
10	—	—	80.1 ± 0.22*
10	10	—	84.2 ± 0.32
10	10	0.25	83.9 ± 0.42
10	10	0.5	84.3 ± 0.38
10	10	1.0	82.5 ± 0.26
10	10	2.0	81.7 ± 0.30

* Standard error of the mean.

the slope of the curve for the adrenalectomized rat is not similar to that obtained for the normal rat it is possible to estimate an increased sensitivity of the order of 4 to 6 fold (Fig. 1). A significant increase in uterine water (82.0%) was obtained with 0.1 G.P.U. of relaxin and a maximum effect (86.2%) was obtained following 100 G.P.U. of relaxin. Treatment with cortisone acetate inhibited the response of the adrenalectomized rat uterus to relaxin. A 44% inhibition was obtained with 0.5 mg. cortisone acetate in rats injected with 10 G.P.U. of relaxin and complete inhibition was obtained with 1.25 mg. cortisone acetate (Table 2).

The simultaneous administration of relaxin and estradiol produced

TABLE 2. UTERINE WATER CONTENT OF THE ADRENALECTOMIZED 24-DAY-OLD RAT 6 HOURS FOLLOWING THE SIMULTANEOUS ADMINISTRATION OF RELAXIN AND CORTISONE ACETATE

No. of animals	Relaxin, G.P.U.	Cortisone acetate, mg.	Uterine water— %
10	—	—	80.1 ± 0.58*
10	10	—	84.9 ± 0.46
10	10	0.5	82.8 ± 0.56
10	10	1.25	80.0 ± 0.50
10	10	2.5	80.9 ± 0.42
10	10	10.0	80.0 ± 0.40

* Standard error of the mean.

varied responses with regard to the uterine water depending on the dosage of each hormone (Fig. 2). The simultaneous administration of 0.005 μg. estradiol and either 0.2 or 0.5 G.P.U. relaxin inhibited the response seen with relaxin alone at that level.

Increasing the dosage of relaxin to 1 G.P.U. caused an additive effect, while further increases in dosage to 2 and 10 G.P.U. produced no greater degree of water imbibition than that observed with relaxin alone. When the level of estradiol was raised to 0.01 μg., the antagonism between the two hormones was apparent only at the lowest dosage of relaxin administered. Each dose above this minimally effective level of relaxin caused an additive result, while the two high relaxin dosages actually produced a synergistic effect on the uterine water response. When 0.02 μg. estradiol was administered, no antagonism was present at any of the concomitantly administered relaxin doses, and in fact, a significant additive effect occurred with 1 and 2 G.P.U. relaxin.

Concentrations of sodium and potassium in the immature rat uterus at definite time intervals following the separate administration of maximally effective doses of relaxin and estradiol are illustrated in Table 3. Following the administration of 0.1 μg. estradiol a rise in sodium from the control level of 59.5 m.eq. to 74.0 m.eq. and a decrease in potassium from 62.0 m.eq. to 40.0 m.eq. occurred at 6 hours. Thirty hours following hormone injection, the sodium level returned to normal, and the potassium con-

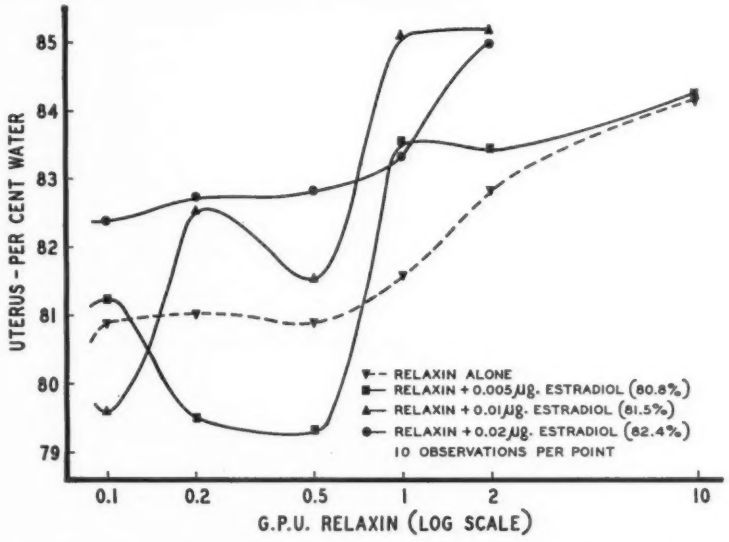

FIG. 2. The effect of simultaneous administration of estradiol and relaxin on the per cent uterine water in the immature rat. Note: Uterine water responses to the three levels of estradiol, when administered alone, are indicated in parentheses.

centration returned to a point approaching the normal level. Six hours after the administration of 50 G.P.U. relaxin there was a rise in sodium to 67.5 m.eq., and at 18 hours a drop to 46.5 m.eq. had occurred. This was accompanied by an increase in potassium from 55.0 m.eq. at 6 hours to

TABLE 3. THE EFFECT OF RELAXIN AND ESTRADIOL ON THE CONCENTRATION OF SODIUM AND POTASSIUM IN THE UTERUS OF THE 24-DAY-OLD RAT

Treatment	Time post injection	No. of animals	Uterine water %	m.eq. Na/Kg. wet wgt.*	% change**	m.eq. K/KG. wet wgt.*	% change**
None	0	5	80.2	59.5	—	62.0	—
Relaxin (50 G.P.U.)	6	5	84.4	67.5	+13**	55.0	−11
Relaxin (50 G.P.U.)	18	5	82.0	46.5	+22**	101.0	+63**
Relaxin (50 G.P.U.)	30	5	79.6	61.5	+ 3	51.0	−21**
Estradiol (0.1µg)	6	5	86.0	74.0	+25**	40.0	−35**
Estradiol (0.1 µg)	30	5	82.2	61.5	+ 3	55.0	−11
None (ADX)	0	5	80.1	54.0	− 8**	110.0	+75**
Relaxin (50 G.P.U.-ADX)	6	5	85.5	49.0	− 8**	90.0	−18**

$$* \text{ m.eq./Kg. Wet Wgt.} = \frac{\frac{\text{m.eq./L.}}{100}}{\text{Wet Wgt.}} \times 10^6.$$

** Indicates significant difference from control value as determined by the Student "t" test at the 1% level of significance. Electrolyte concentrations of the adrenalectomized animals which received relaxin were compared with those of the adrenalectomized controls. All others were compared with electrolyte concentrations of the intact controls.

101.0 m.eq. at 18 hours. As is the case with estradiol, both sodium and potassium levels had returned to points approaching normal, 30 hours after relaxin administration. In adrenalectomized animals the potassium level was significantly higher than in the intact controls, while the sodium concentration was slightly lower. Both underwent a decrease 6 hours following the administration of relaxin.

DISCUSSION

The present results indicate a similarity in action of the two hormones, estradiol and relaxin, in their ability to cause an increased uterine water content in the rat. These data confirm the finding that relaxin can produce this effect without the necessity of previous priming with estrogen (3). In a series of other effects produced by relaxin, such as separation of the pubic symphysis of the guinea pig and mouse (6, 7), dilation of the uterine cervix in the cow and sow (8, 9), and inhibition of uterine contractions in the rat (10, 11), it was necessary to first sensitize the responsive tissue with estrogen.

The role of the adrenal cortex in this phenomenon has heretofore been rather neglected. Szego and Roberts, (12), in studying pituitary-adrenal cortical relationships to the uterine water phenomenon, found that adrenalectomy significantly enhanced the response to estrogen. Our results have indicated a similar role of the adrenal cortex in reducing the uterine water response to relaxin administration. The sensitivity of the uterus to relaxin is increased by a factor of 4 to 6 following adrenalectomy. The minimal effective dose of the hormone is decreased and the maximum response is increased. Further evidence in support of an effect by the adrenal cortex in this phenomenon is illustrated by the finding that administration of 0.5 mg. cortisone acetate partially inhibits the uterine water increase observed following 10 G.P.U. relaxin, while 1.25 mg. cortisone acetate completely blocks the response. Progesterone also produced a partial inhibition of the action of relaxin on the uterine water. In this respect both cortisone and progesterone had the same action in preventing or partially inhibiting the increased uterine water content following relaxin or estradiol (12).

The mechanism of action of both estradiol and relaxin in causing the uterine water increase is at present not clear, but the depressant action of the adrenal cortex would appear to suggest that the electrolytes may be involved in this phenomenon. Talbot et al. (13) correlated a rise in sodium concentration with the increased uterine water content, at 6 hours following estrogen administration and demonstrated a rise in potassium content at 30 hours, which was the time at which a secondary rise in percent water and dry weight of the uterus occurred. Our findings indicate that the electrolyte pattern following relaxin administration generally follows the picture observed following estrogen.

There appears to be no doubt that a similarity in action exists between

estradiol and relaxin. Furthermore, an actual potentiating action of relaxin with estrogen has been noted in the rat (14), and in the mouse (15). The present results indicate that the simultaneous administration of estradiol and relaxin may lead to an inhibition of the effect seen with relaxin alone or to a synergism. While minimally effective doses of estradiol and relaxin cause a depression of uterine water content, other dosages show either an additive effect or no effect, different from that observed following either hormone, when administered alone. There appears to be no definite ratio of these hormones at which synergism results, but the data definitely indicate a true complemental action.

It is obvious that the relationship of the two hormones in their action on uterine water content of the rat is not a simple one.

REFERENCES

1. Astwood, E. B.: *Amer. J. Physiol.* **126**: 162. 1939.
2. Astwood, E. B.: *Endocrinology* **23**: 25. 1938.
3. Zarrow, M. X. and D. M. Brennan: *Proc. Soc. Exp. Biol. and Med.* **95**: 745. 1957.
4. Steinetz, B. G., V. L Beach, R. P. Blye and R. L. Kroc: *Endocrinology* **61**: 287. 1957.
5. Zarrow, M. X., E. A. Lazo-Wasem and R. L. Shoger: *Science* **118**: 650. 1953.
6. Hisaw, Frederick L.: *Proc. Soc. Exp. Biol. and Med.* **23**: 661. 1925.
7. Hall, Kathleen: *Endocrinology* **5**: 174. 1947.
8. Graham, E. F. and A. E. Dracy: *J. Dairy Sci.* **35**: 499. 1952.
9. Zarrow, M. X., G. M. Neher, D. Sikes, D. M. Brennan and J. F. Bullard: *Amer. J. Obstet. and Gynecol.* **72**: 260. 1956.
10. Felton, L. C., E. H. Frieden and H. H. Bryant: *J. Pharmacol. Exp. Therap.* **107**: 160. 1953.
11. Krantz, J. C., H. H. Bryant and C. J. Carr: *Surg. Gynec. Obstet.* **90**: 372. 1950.
12. Szego, C. M. and S. Roberts: *Amer. J. Physiol.* **152**: 131. 1948.
13. Talbot, N. B., D. H. Lowery and E. B. Astwood: *J. Biol. Chem.* **132**: 1. 1940.
14. Hamolsky, M. and R. C. Sparrow: *Proc. Soc. Exp. Biol. and Med.* **60**: 8. 1945.
15. Dewar, A. D., K. Hall and W. H. Newton: *J. of Physiol.* **105**: 37. 1946.

ENDOGENOUS AND EXOGENOUS VASOPRESSIN ON ACTH RELEASE[1]

BUFORD NICHOLS, JR.[2] AND ROGER GUILLEMIN

Department of Physiology, Baylor University College of Medicine, Houston, Texas

ABSTRACT

Simultaneous studies of the release of antidiuretic hormone (vasopressin, ADH) and corticotrophin (ACTH) were performed in unanesthetized trained dogs. Antidiuresis was used as the index of ADH liberation and plasma free 17-hydroxy-corticosteroid levels were used to indicate ACTH release. No correlation was observed between discharge of ACTH and release or inhibition of the release of ADH. Further studies with highly purified vasopressin showed that exogenous ADH was unable to stimulate the secretion of ACTH until a threshold of $3–7 \times 10^3$ times the dose reproducing maximal physiologic antidiuresis was exceeded. The physiological significance of the data evidencing ACTH release upon injection of vasopression is debated and questioned.

VASOPRESSIN (ADH) has been proposed as the ultimate mediator of ACTH release on the grounds of several arguments based on inferential conclusions (1) and on the observation that injection of large quantities of the pressor antidiuretic fraction of the posterior lobe of the pituitary stimulates release of ACTH (2, 3, 4, 5, 6). In accordance with this hypothesis, one would expect that acute release of endogenous ADH should associate with observable signs of increased ACTH secretion. Were this not the case, a strong argument would have been procured against the vasopressin theory. To study the validity of this assumption, ADH release as ascertained by the degree of ensuing antidiuresis was induced by intracarotid injections of hypertonic saline in conscious trained dogs and pituitary-adrenal function was explored simultaneously by measurement of plasma free 17-hydroxy-corticosteroid levels. The minimal amount of highly purified vasopressin that would produce ACTH release upon injection in the carotid was also determined and related to amounts of endogenous vasopressin referable to physiological antidiuresis.

MATERIAL AND METHODS

Preparation and care of the animals. Three female dogs weighing 14, 15 and 18 kg. were prepared with a carotid loop and trained for intracarotid injections and measurement of diuresis in the conscious state according to the methods of Verney (7).

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Measurement of diuresis and stimulation of antidiuresis. The technique chosen for hydration was that of van Dyke (8) which requires two successive water loads by stomach tube. During the second load, urine was collected by means of indwelling catheters at 5 minute intervals; the volume was recorded as average flow per minute per period of collection. Endogenous ADH release was obtained by intra-carotid injection of hypertonic saline (4 to 8%) prepared and administered according to Verney (7) i.e. total volume of 2.0 ml. injected over 5–20 seconds. The per-cent-change of urine flow from the pre-injection level to the lowest flow observed after injection (% antidiuresis) was used as a convenient quantitative expression of the antidiuresis obtained. Lysine-vasopressin (LVP) was prepared by the method of Ward and Guillemin (9) and further purified by electrophoresis. The doses of LVP injected ranged from 0.066 to 2.12 units. These were injected in 2 ml. of 0.9% saline into the carotid artery over a period of 60 seconds. The specific activity of the preparation used was 260 u./mg. when assayed against the USP Posterior Lobe Reference Standard in the rat treated with dibenamine (26).

Assessment of ACTH release. ACTH release was assessed by serial measurements of the plasma free 17-hydroxy-corticosteroid (17OHC) levels. 17OHC were determined by the method of Nelson and Samuels (10) on 20 ml. blood samples. Blood samples were taken from the carotid artery immediately before and 15 and 60 minutes after the injection of hypertonic saline or LVP. The experiments were planned so that no more than 120 ml. of blood were drawn within a single week from each animal. All samples were collected between 11:30 A.M. and 2:00 P.M. With this method, our range for normal (resting) concentrations of 17OHC is 0 to 8 μg./100 ml. of plasma. These values are based on approximately 150 single determinations; they are in agreement with what has been reported in the literature using the same method and on plasma samples obtained at similar times of the diurnal rhythm (30).

RESULTS

Endogenous ADH

a. *Inhibition of release.* (Fig. 1). During the training procedure, a number of blood samples were drawn from the dogs in various stages of the period of hydration and diuresis. When these 17OHC concentrations and the values at Time zero (i.e. at time of maximal diuresis) in subsequent experiments were plotted against the urine flow per minute, no correlation between the variants was evident. Inhibition of the release of ADH can thus be obtained simultaneously with evidence of stimulation of the release of ACTH.

b. *Stimulation of release.* (Table 1). The amounts of hypertonic saline administered were limited by the intrinsic irritant effects of the hypertonic solution for each dog, as some discomfort was observed in most instances (7). This was manifested by smacking of the lips, whining, stretching of the extremities and occasional mild struggling. In 10 experiments the antidiuresis so obtained varied from moderate to maximal. In 8 of these experiments no change of the corticoid levels was observed over the 60 minutes of study, irrespective of the antidiuresis produced. In 2 of these experiments, increased plasma 17OHC levels were observed; in both of these, technical errors had provoked extremely painful reactions of the animals. In one of these experiments, most of a 2 ml. dose of 8% saline was ex-

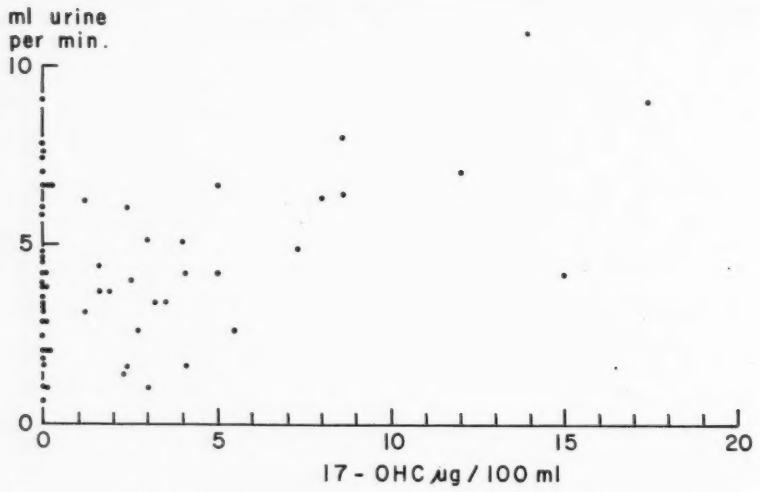

FIG. 1. Scattergram of 17OHC related to urine flow.

travasated; there was only a minimal antidiuretic response. In the other, the 2 ml. of 8% saline were injected within 5 seconds. the dog leaped off the table and howled in obvious reaction to acute pain.

Exogenous ADH. (Table 2). The injection of LVP was always followed by maximal antidiuresis, blanching of the gums, and marked bradycardia. A very distinct rise in the 17OHC levels was observed with the larger doses. A threshold for ACTH release was found in each dog. In 2 animals it was

TABLE 1. ENDOGENOUS ADH. INJECTION OF HYPERTONIC SALINE IN THE CAROTID ARTERY. MEASUREMENT OF ANTIDIURESIS AND LEVELS OF PLASMA 17OHC (TEN TRIALS IN 3 DOGS)

Time of sampling	Plasma 17OHC concentrations in µg./100 ml.									
0	0	0	8.6	0	8.6	0	0	0	0	8.0
15 minutes	0	0	8.2	0	7.3	—	—	0	0.2	16.4
60 minutes	0	0	7.3	0	—	0	0	0	16.7	14.5
% Antidiuresis	48	51	34	69	100	78	81	88	100	21

TABLE 2. EXOGENOUS ADH. INJECTION OF LYSINE-VASOPRESSIN IN THE CAROTID ARTERY. MEASUREMENT OF ANTIDIURESIS AND LEVELS OF PLASMA 17OHC (13 TRIALS IN 3 DOGS)

Time of sampling	Plasma 17OHC concentrations in µg./100 ml.												
0	4.0	0	0	0	0	0	2.5	4.1	2.6	1.9	0	0	0
15 minutes	2.5	0	0	2.1	0	0	5.4	5.6	3.7	4.6	5.5	5.8	2.0
60 minutes	0	0	0	6.0	0	0	2.5	4.1	8.2	0	4.7	3.2	6.2
Doses of LVP in USP Units	.066	.066	.066	.130	.130	.130	.260	.260	.260	.520	.520	1.4	2.12
% Antidiuresis	100	100	100	100	100	100	100	100	100	100	100	100	100

0.260 U. while in the other, stimulation of ACTH release was obtained with 0.130 U. The antidiuresis was so profound in each experiment that comparison of the antidiuretic effects of the different doses of LVP was difficult to assess. In those experiments where a recovery of diuresis was evident before exhaustion of the water load, the time between the onset of antidiuresis and a 50% recovery was calculated. These figures indicated a quantitative relationship between dose and duration of antidiuresis but no correlation with the pituitary-adrenal stimulation. The stimulation of ACTH release appeared as an all-or-none type of response within the range of doses of LVP administered. There was no relation between the dose of LVP and the 15 or 60 minute response once the threshold of stimulation of ACTH release had been exceeded. The most common response consisted of a peak at 15 minutes, followed by a fall at 60 minutes. In three experiments the 15 minute peak was not observed but an almost linear climb to a high 60 minute response was observed. The pattern of the response was not related to the dose of vasopressin administered.

DISCUSSION

It might be argued that a lack of correlation between degrees of antidiuresis and levels of plasma 17OHC in the dog, a species where these are usually very low, is only due to a large difference in sensitivity of the two phenomena to their respective affectors.

The data available in this paper do not provide a quantitative answer to this question. However, numerous observations of increased levels of 17OHC upon stimulation of *endogenous* ACTH release have been obtained in this series of experiments. (Fig. 1, Tables 1 and 2). It is interesting that these increases in plasma 17OHC levels bore no relationship to the degree of antidiuresis observed concomitantly: some of the highest levels of 17OHC observed were found in the maximal *diuresis* of the water loading (Fig. 1) during the training period of the animals when it was an obviously "stressful" procedure to them.

Jewell and Verney have reported (11) that the endogenous release of ADH to produce maximal physiological antidiuresis is in the range of 12×10^8 molecules/second. Knowing the specific activity of the lysine-vasopressin used in these experiments at 260 units/mg., the molecules/second administered can be calculated. Thus the threshold for ACTH stimulation is 4.2×10^{12} to 8.6×10^{12} molecules/second. This indicates a factor of 3.5×10^3 to 7.1×10^3 between the amount necessary for maximal antidiuresis and the threshold dose for ACTH stimulation. If only one-thousandth (1×10^{-3}) of the dose injected into the carotid artery arrived at the receptor site for ACTH release, the above discrepancy could perhaps be explained on the basis of dilution alone. However, by the same reasoning, the endogenous ADH would have to reach this level to stimu-

late ACTH release. This would dictate a maximal antidiuresis each time ACTH is released. This is not supported in this series of experiments by the high levels of 17OHC found during *diuresis* (Fig. 1) or by this observation of a rise in 17OHC concentration with only minimal antidiuresis (Table 1).

The administration of pressor extracts of the posterior lobe of the pituitary (Pitressin, Protopituitrin) or of highly purified vasopressins of natural or synthetic origin has been reported to produce stimulation of the pituitary-adrenal system (2, 3, 4, 5, 6). If this would appear of questionable meaning in the normal subject (3, 4, 6), the fact observed in preparations in which stress induced ACTH release was impaired or prevented (2, 5, 12) has been taken as strong support of the theory proposing ADH as the physiological mediator of ACTH release. In reaction with this conclusion, it was pointed out (21, 25, 27) that the doses of vasopressin used in the experiments of these various authors are so large that they are of little significance to any mechanism underlying physiological phenomena. Indeed, explanations other than the above apparently obvious conclusions have begun to appear regarding the mechanism of action of these pharmacological doses of vasopressin (13, 14, 15). The results reported here have confirmed that highly purified vasopressin can produce pituitary-adrenal stimulation. This was done by injecting in the carotid doses of the material greatly in excess of those reproducing physiological antidiuresis. Conversely, controlled release of endogenous ADH was never followed by evidence of ACTH release whereas definite signs of pituitary adrenal stimulation were obtained in conditions corresponding to no stimulation or even inhibition of ADH release (Fig. 1). A similar conclusion was recently reached by McDonald (16) in contradistinction to his previous position (3, 4). Only a highly speculative theory could reconcile these data and the postulated role of vasopressin as the ultimate mediator of ACTH release. It would involve a differential release of vasopressin from the posterior lobe, to the periphery for antidiuresis, or to the anterior pituitary for ACTH secretion (by way of the short vessels which have been described recently (17, 18, 19) or by the hypothalamo-hypophysial portal vessels). This hypothesis has indeed been ventured by McCann in private discussions. The physiological complications inherent to this concept are rendered even more unnecessary by the recent demonstration (20, 29) that stimulation of ACTH release can be obtained with a linear dose-response relationship by *peripheral* administration of crude CRF preparations (Guillemin's fraction D), whereas equipressor doses of purified lysine vasopressin given by the same route are completely ineffective in stimulating release of ACTH. Stimulation of the discharge of ACTH by *endogenous* vasopressin may still be possible in these unusual stresses such as massive sudden blood loss where unusually large amounts of ADH appear to be released suddenly (22). Stimulation of the discharge of ACTH by *exogenous* vaso-

pressin will always be possible if large enough doses of the material are administered. Since this is true *in vitro* (23, 24) as well as *in vivo* in animals with hypothalamic lesion or blockade (5, 13, 20, 29), it may be best explained as previously proposed (14, 23) by an overlapping of activities of molecules closely related structurally as seems to be probable for CRF and vasopressin.

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REFERENCES

1. Mirsky, I. A., M. Stein and G. Paulisch: *Endocrinology* **55**: 28. 1954.
2. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exper. Biol. and Med.* **87**: 318. 1954.
3. McDonald, R. K. and V. K. Weise: *Proc. Soc. Exper. Biol. and Med.* **92**: 481. 1956.
4. McDonald, R. K., V. K. Weise and R. W. Patrick: *Proc. Soc. Exper. Biol. and Med.* **93**: 348. 1956.
5. McCann, S. M.: *Endocrinology* **60**: 664. 1957.
6. Martini, L., A. de Poli and S. Curri: *Proc. Soc. Exper. Biol. and Med.* **91**: 490. 1956.
7. Verney, E. B.: *Proc. Roy. Soc. London* **135**: B:25. 1947.
8. Van Dyke, H. B., K. Adamsons, Jr. and S. L. Engel: *Recent Progress in Hormone Research* **11**: 1. 1955.
9. Ward, D. N. and R. Guillemin: *Proc. Soc. Exper. Biol. and Med.* **96**: 568. 1957.
10. Nelson, D. H. and L. T. Samuels: *J. Clin. Endocrinol.* **12**: 519. 1952.
11. Jewell, P. A. and E. B. Verney: *Phil. Tr. Roy. Soc. London* **240**: 197. 1957.
12. Sayers, G.: *Fed. Proc.* **15**: 162. 1956.
13. Royce, P. C. and G. Sayers: *Proc. Soc. Exper. Biol. and Med.* **98**: 70. 1958.
14. Guillemin, R.: *Endokrinologie* **34**: 193. 1957.
15. Hume, D. M. and D. H. Nelson: *Program of The Endocrine Soc.* 1957 Meeting, p. 98.
16. McDonald, R. K., H. N. Wagner, Jr. and V. K. Weise: *Proc. Soc. Exper. Biol. and Med.* **96**: 652. 1957.
17. Landsmeer, J. M. F.: *Acta Anat.* (Basel) **12**: 83. 1951.
18. Jewell, P. A.: *J. Endocrinol.* **14**: xxiv. 1956.
19. Duvernoy, H.: Thesis, Faculté de Médecine, Paris, 1958.
20. Guillemin, R., G. W. Clayton, J. D. Smith, H. S. Lipscomb, D. N. Ward and A. V. Schally: *Program of the Endocrine Soc.* 1958 Meeting, p. 37.
21. Guillemin, R.: *Rev. Suisse Zool.* **64**: 673. 1957.
22. Ginsburg, M. and L. M. Brown: in "The Neurohypophysis" edited by H. Heller, Academic Press, New York, p. 109. 1957.
23. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Housholder: *Endocrinology* **60**: 488. 1957.
24. Schally, A. V., M. Saffran and B. Zimmermann: *Biochem. J.* **70**: 97. 1958.

25. NICHOLS, B., JR., R. GUILLEMIN AND R. A. SEIBERT: *Fed. Proc.* **17**: 398. 1958.
26. DEKANSKI, J.: *Brit. J. Pharmacol.* **7**: 567. 1952.
27. GAUNT, R., C. W. LLYOD AND J. J. CHART: in "The Neurohypophysis" edited by H. Heller, Academic Press, New York, p. 233. 1957.
28. RYDIN, H. AND E. B. VERNEY: *Quart. J. Exp. Physiol.* **27**: 343. 1938.
29. GUILLEMIN, R., B. NICHOLS, JR. AND H. S. LIPSCOMB: *C. R. Acad. d. Sc.* (Paris). **247**: 1664. 1958.
30. HARWOOD, T. C. AND J. W. MASON: *Am. J. Physiol.* **186**: 445. 1956.

DIURNAL VARIATION IN RESPONSE TO GONADOTROPIN IN THE MOUSE

D. R. LAMOND[1] AND A. W. H. BRADEN[2]

Department of Veterinary Physiology, University of Sydney, Sydney, Australia

ABSTRACT

With the use of immature mice 18–20 days of age it was shown that a single injection of 0.1–10.0 I.U. chorionic gonadotropin (HCG) or pregnant mare serum gonadotropin (PMS) resulted in greater uterine and ovarian weights 24 hours later if given in the afternoon as compared with the morning. A diurnal difference in response was also observed in ovarian but not in uterine weights at 48 hours after injection. The diurnal variation in response was not apparent in mice aged 14–17 days, but was significant in 6 week old mice. In the latter the period of increased responsiveness was more restricted than in mice aged 20–22 days.

A study of the time-response relationships indicated that the greater response in organ weights at 24 hours was a consequence of an earlier beginning of growth rather than an increased rate of growth. Hypophysectomy and high doses of PMS and HCG (30 I.U.) prevented the diurnal variation in response, which was also not apparent in the uterine weight response to a single injection of either oestradiol, post-menopausal urine gonadotropin or a crude extract of anterior pituitary glands.

It is concluded that there is a diurnal variation in pituitary function and not in ovarian or uterine function. The results are discussed in relation to the gonadotropic hormones.

IN STUDIES in this laboratory on the mode of action of various gonadotropin preparations on the mouse ovary, the two responses examined have been ovarian weight and uterine weight. Previous work (12, 14), employed a single injection technique in which the gonadotropin was administered subcutaneously in isotonic saline 44–46 hours before killing.

Before this work could be extended to multiple injection techniques, particularly spread over one day, it was thought important to investigate the possibility of diurnal variation in response to a single injection. This was prompted by the relationship known to exist between oestrus and ovulation and the diurnal rhythm of light and dark in mice and rats (1, 3, 9, 17). Braden (2) has extended this work to show that the time of ovulation

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[1] Present address: Faculty of Rural Science, University of New England, Armidale, N.S.W., Australia.

[2] An Officer of the Division of Animal Health and Production, Commonwealth Scientific and Industrial Research Organization. Present address: Sheep Biology Laboratory, Prospect, N.S.W., Australia.

in mice is affected by changes in the relative length of the light and dark phases of the 24 hour cycle. In studies on the release of the ovulating hormone in rats, Everett and Sawyer (6, 7) have presented evidence which points to the presence of a diurnal rhythm in the excitability of the neural component of the "LH-release apparatus." They have suggested that between 2 and 4 P.M. each day there was in their rats a characteristic excitability of the neural component of the ovulating mechanism and that the particular night during which oestrus and ovulation took place was determined by the interaction of this diurnal pattern and the oestrogen and progesterone produced by the developing follicles.

Preliminary work indicated that there is a diurnal rhythm in the sensitivity of the immature mouse to gonadotropin. The experimental approach was, firstly, to establish clearly this fact, and secondly, to determine the extent of the period of increased sensitivity. In addition, experiments were carried out to compare the time-response relationships in both uterine and ovarian weights to morning and afternoon injections, and to gain some indication of the mechanism of the diurnal rhythm.

MATERIALS

Animals. Immature female albino mice were weaned when 18–20 days old, except where otherwise indicated (Experiment IV). Injections commenced as soon as practicable after weaning. Large groups of mice of similar age were available for each experiment; the method of breeding has been previously described (4). In all experiments the mice were allotted to experimental groups entirely at random. They were housed five mice per cage, each cage containing mice on a particular treatment. The conditions in the animal house were held constant at $78 \pm 2°$ F, 14 air changes per hour, and light: dark ratio of 12:12 hours (lights, fluorescent white, on 6 A.M.–6 P.M.).

All injections of gonadotropins, dissolved in isotonic saline, were made subcutaneously in the dorsal region. The injection volume was 0.1 ml. The mice were killed by cervical dislocation and the body weights recorded immediately. The uteri and ovaries were dissected free of extraneous tissue under a binocular dissecting microscope and weighed on a torsion balance after pressing on filter paper. The uteri were weighed to the nearest 0.5 mg. and the ovaries to the nearest 0.25 mg.

Gonadotropins. The gonadotropic substances used in the majority of experiments were human chorionic gonadotropin (HCG)[3] and pregnant mare serum gonadotropin (PMS).[2] A large number of vials of the same batch of each substance was available.

EXPERIMENTS AND RESULTS

Experiment I

The experimental design was a $2 \times 4 \times 2 \times 3$ plus a $2 \times 4 \times 2 \times 2$ factorial. In the first, two gonadotropins, each at four dosage levels, were given in a single injection at either 9 A.M. or 9 P.M. This experiment was performed on three successive days. The second experiment, carried out concurrently with the first, was the examination of the effect of storage of gonadotropin

[3] Pregnyl and Gestyl obtained from Organon Laboratories Ltd., London.

over the period of the test. The total number of treatment combinations was 48 plus 32, of which 16 were common to both experiments. Since each group comprised five mice, a total of 320 mice were involved in the two experiments (Table 1). The mice were killed 24 hours after injection.

An important consideration was the possible bias due to time from weaning to infection. This was overcome by allotting mice at random to each of the six times of injection (2 times per day for 3 days) prior to commencement of the experiment. Each batch of mice was then weaned twelve hours before the appropriate time.

The dosage levels of gonadotropin were 0.22, 0.66, 6.6 and 20 nominal I.U. HCG and 0.11, 0.33, 3.3 and 10 nominal I.U. PMS. The two lowest

TABLE 1. SCHEDULE OF OPERATIONS IN EXPERIMENT 1

	Mice weaned	Mice injected	Mice killed
Day 0 9 P.M.	40		
Day 1 9 A.M.	40	40	
9 P.M.	40	40	
Day 2 9 A.M.	40	40	40
9 A.M.	80	40	40
Day 3 9 A.M.	80	80*	40
9 P.M.		80*	40
Day 4 9 A.M.			80
9 P.M.			80

* 40 mice were injected with fresh gonadotropin and 40 were injected with the preparations given on Day 1 and stored for 48 hours.

levels of each substance were expected to give responses which fell on the linear portion of the log dose-log uterine response curves (4) and the two highest levels, responses which were on the linear portion of the log dose-ovarian weight response curves (12).

Immediately prior to injection a vial of gonadotropin was dissolved in the appropriate amount of isotonic saline to give a solution containing the highest dosage level in 0.1 ml. The lower levels were prepared by dilution. The sixteen solutions (two gonadotropins $\times$ four levels $\times$ two times of the day) employed on the first day were stored in the refrigerator and used again 48 hours later.

The uterine and ovarian data were analysed separately but the statistical methods were similar. The regressions of response on body weight were calculated for eight groups of 20 mice; i.e., the six groups that received the fresh preparation over the three days and the two groups that received the stored preparations on Day 3. The regression coefficients were examined for evidence of heterogeneity, $\chi^2_{(7)} = 13.62$, $0.05 < P < 0.1$ (uterine weights) and $\chi^2_{(7)} = 9.91$, $0.1 < P < 0.2$ (ovarian weights). These results indicated that an overall regression could be used. Hence the group totals were cor-

rected for concomitant variation due to body weight and the corrected totals were used in the analysis.

The value of the regression of log uterine response on log body weight, $b=1.12$, and the value of the corrected within-group variance, $EMS_c=71.3$, were similar to previous results (4). In addition the value for the regression of ovarian response on body weight, $b=0.32$, and the corrected within-group variance, $EMS_c=0.37$, are typical of results in this laboratory.

The data were analysed in two steps.

(i) Comparisons were made between preparations, doses, times of day and replications using the fresh preparations.

(ii) Comparisons were made between preparations, doses, times of day and stored versus fresh materials for the results of the injections carried out on Day 3.

The analyses of variance are presented in Table 2 (Appendix) and the results are shown graphically in Figure 1.

Uterine weight. The mean response to HCG was significantly greater than to PMS. The slopes of the log dose-log response lines were similar except for a significantly lower slope for the HCG at the evening injection, which occurred because in the evening the lowest level was sufficient to give a maximal response. Time to time differences in response, that is time of day and replications, were significant and there was no interaction

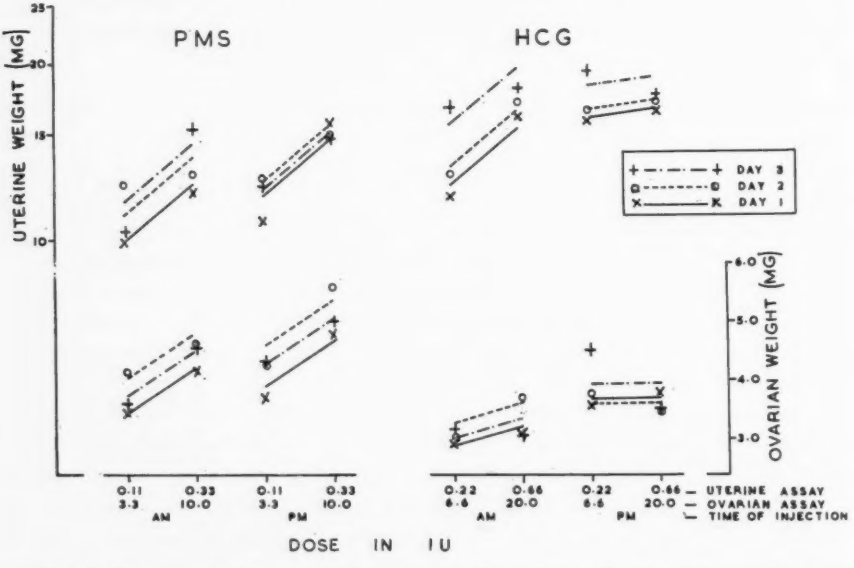

FIG. 1. The results of Experiment 1; showing diurnal variation in uterine and ovarian weights 24 hours after an injection of either PMS or HCG. Three replicates on successive days.

between the two, but it cannot be concluded from the analysis in Table 2 that differences in response between morning and evening injections were not entirely due to a linear time trend in sensitivity of the test animals. Hence it was important to examine the data further to establish the extent of time trends which were independent of time of day trends. This was done by estimating the regressions of time on response for each of the two sources of variation and comparing them in a t-test. There was a significant difference in response between morning and evening injections in spite of the underlying increase in sensitivity over the three day period ($t_{(127)}=2.24$; $P<0.05$).

The effect of storing the solutions in a refrigerator for 48 hours was a significant reduction in the response of the HCG preparations only, indicating deterioration over the period. Lamond (11) and Claringbold and Lamond (4) could not find evidence of deterioration in HCG substances when kept for much longer periods than in this experiment. However the preparations were stored at high concentrations whereas the solutions which deteriorated in the present experiment were at low concentrations.

Ovarian weight. Again it was apparent that there was no interaction between time of day and replication and as with the uterine responses it has been possible to show that there was a significant diurnal difference in response ($t_{(127)}=2.69$, $P<0.01$). No significant difference was found between the stored and fresh materials on Day 3.

Experiment II

In Experiment I the mice were killed 24 hours after the injection of gonadotropin—a time when both uteri and ovaries would be in the rapid growth phase. In view of the greater response in uteri and ovaries which resulted from the evening injection of either gonadotropin, it was thought advisable to determine if this were due merely to a faster initial rate of growth or to an actual augmentation of the final response. The uterine weight response is maximal by 40–48 hours (4) and the ovarian weight response to HCG and PMS at the dosage levels used in this experiment is maximal by 40–60 hours (unpublished data). Structural changes such as the formation of haemorrhagic follicles and corpora lutea occur in the ovaries after 60 hours. Therefore, in Experiment II the same four levels of each gonadotropin were administered to groups of randomly selected mice (five per group) which were then killed 24 or 48 hours later. The mice were weaned one hour before injection and the injections were given at 8–30 P.M. on one day and 8–30 A.M. the next day.

The data for both uterine and ovarian weights at 24 and at 48 hours were analysed separately; the experimental design thus consisted of four 4×2 factorials carried out concurrently. As in Experiment I the group totals were corrected for concomitant variation due to body weight differences and the corrected totals used in the analyses of variance which are sum-

marized in Table 3 (Appendix). The results are shown graphically in Figure 2.

Uterine weights. At 24 hours and 48 hours the mean response to HCG was greater than to PMS. This indicated that the levels selected were relatively more potent than the corresponding levels of PMS. The slope of the

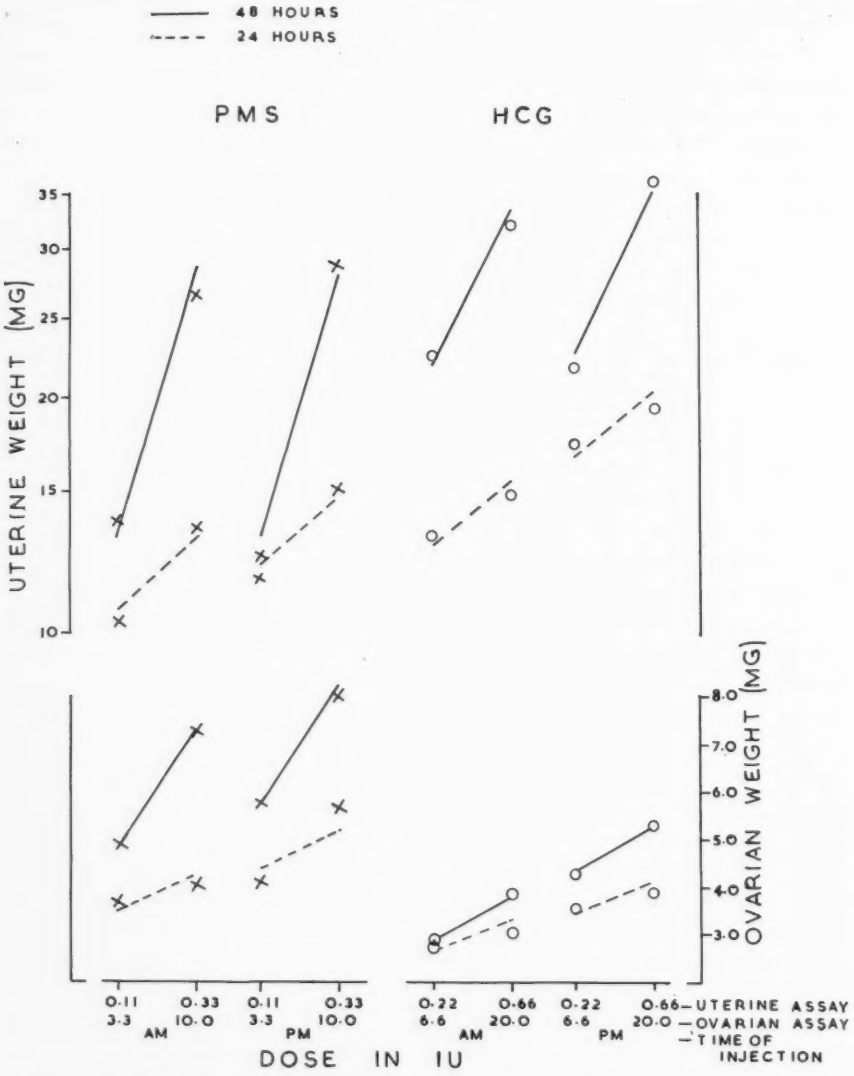

FIG. 2. The results of Experiment 2; showing diurnal variation in both uterine and ovarian weights at 24 hours, but not in uterine weights at 48 hours after an injection of PMS or HCG.

log dose-log response lines at 24 hours were similar, and as in Experiment I, the evening response was significantly greater than the morning response for both PMS and HCG. However by 48 hours when the uteri might be considered to have ceased growing there was no significant difference for either substance. Since all mice were injected over the same period of time it is clear that time changes in sensitivity, apart from the evening versus morning differences, cannot have accounted for significant differences at 24 hours followed by no differences at 48 hours.

Ovarian weights. At 24 hours the mean response to PMS was significantly greater than to HCG, and the response to the evening injection of both substances was greater than to the morning injection. No difference in the slope of the log dose-ovarian weight response lines was demonstrated. At 48 hours the mean response to PMS was much greater than to HCG and the slope of the dose-response lines was also steeper. There was a significantly greater response to the evening injection of both gonadotropins.

Experiment III

The extent of the period of altered response in various groups of mice injected at different times of the day was examined; the mice were killed 24 hours after the injection. Mice of three different ages were used in three experiments carried out in successive weeks.

(a) Mice 14–17 days old, weighing 8–11 gm. were injected immediately after weaning. The times of injection were 9 A.M., 1 P.M., 5 P.M., 9 P.M., 1 A.M. and 5 A.M. Two dosage levels of PMS, 0.5 I.U. and 5.0 I.U. were given to groups of five mice at each time.

(b) Mice 20–22 days old, weighing 10–14 gm., were injected immediately after weaning. The times of injection were the same as those in III (a). Two dosage levels of PMS, 1.0 I.U. and 10.0 I.U., were given to groups of five mice at each time.

(c) Mice 40–42 days old, weighing 13–17 gm., that had been weaned approximately three weeks prior to the experiment were used. One level of PMS, 1.0 I.U. was given to groups of 7 mice at 8–30 A.M., 12–30 A.M., 4–30 P.M., 8–30 P.M. and 12–30 P.M.

The body weights referred to were the weights recorded at slaughter. In (a) and (b) these weights were similar to weaning weights whereas in (c), in order to reduce variability in response (unpublished observations), the mice were without food for the 24 hours of the test. Since these mice lost an average of 2 gm. in this period the actual weight at commencement would have been 15–19 gm.

Both uterine and ovarian weights were recorded for each mouse. The data were analysed as in the previous experiments, the analyses of variance are summarized in Table 4 (Appendix) and the results are shown graphically in Figure 3. The results refer only to the uterine weights since the ovarian weights gave similar results.

Mice 14–17 days old did not show significant diurnal differences in response in either uterine or ovarian weights. Slightly older mice, however, exhibited a period of altered responsiveness in uteri and ovaries extending over the twelve hours from midday to midnight. In the mice which were approaching puberty the period of altered sensitivity was reduced to a few hours in the mid-afternoon. It should be mentioned that the ovaries in the youngest mice responded poorly; they were only slightly above control values even with the higher dosage. This undoubtedly accounted for the

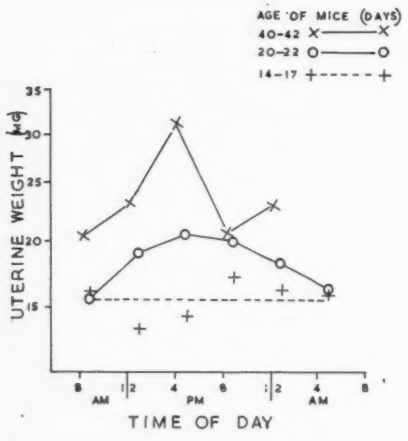

FIG. 3. The effect of age on diurnal variation in uterine weight response 24 hours after an injection of PMS.

dose difference in the uterine response in these mice, whereas in mice a few days older the ovaries responded well to both levels and the uterine weight responses were maximal.

Experiment IV

It seemed likely from the results of Experiment II that the diurnal differences in the responses observed at 24 hours might have been due to an earlier start of growth following the evening injection. An attempt was made to test this hypothesis by establishing the time-response relationships for both uterine and ovarian weights over the period of rapid growth. Two experiments were conducted:

(a) Groups of five mice were injected with 1.0 I.U. or 10.0 I.U. PMS and killed 15, 20, 25 and 30 hours later. The times of injection were 9 A.M. and 9 P.M. the day after the mice were weaned.

(b) Groups of four mice were injected with 1.0 I.U. or 10.0 I.U. HCG and killed 15, 20, 25 and 30 hours later. The times of injection were 4 P.M. the day of weaning and 6–30 A.M. the following day.

The analyses of variance are summarized in Table 5 (Appendix) and the results shown graphically in Figure 4. The mean uterine response over the

period 15–25 hours for HCG and 20–30 hours for PMS was significantly greater to the evening injection than to the morning injection. The time-log uterine weight lines were linear and parallel in each case. Thus the uteri commenced rapid growth earlier after evening injection and this advantage was apparently maintained throughout the greater part of the growth period. It is of interest that after an injection of HCG the rapid growth phase appeared to commence earlier than after PMS. Since the experiments were conducted on different days it is not possible to be cer-

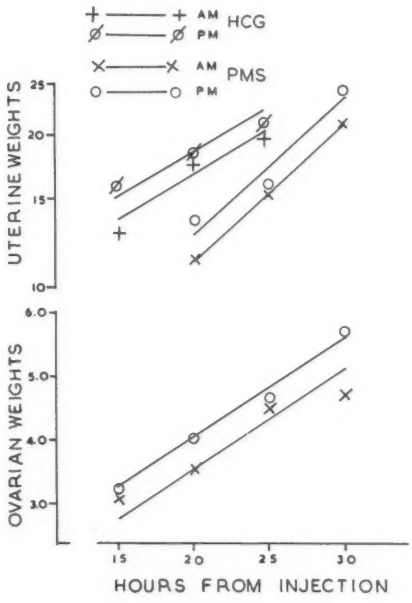

FIG. 4. Time-response relationship for uterine and ovarian weights following a single injection of HCG or PMS.

tain of this, however. There were no significant differences in the responses to the dosage levels for each gonadotropin, each being maximal for uterine weight, hence the data were combined for the purposes of graphical representation in Figure 4.

Only the data for the higher dosage level of PMS, (10.0 I.U.), have been included in the analysis of ovarian weights. The responses to 1.0 I.U. PMS and 1.0 I.U. HCG were minimal, and the ovarian responses to 10.0 I.U. HCG were not sufficiently great to show significant effects with four mice per group. The response to 10.0 I.U. PMS was apparently linear over the period examined, and as with the uterine weights, the ovarian weight response to the evening injection was significantly greater than that to the morning injection, indicating that there was less delay before the commencement of ovarian growth after the evening injection.

Experiment V

It was of some interest to determine if the diurnal variation in ovarian and uterine response was due to a diurnal rhythm of ovarian and perhaps also, uterine, responsiveness. These possibilities were examined in the following way:

(a) Forty-five mice, 18–20 days old and 10–12 gm. body weight, were hypophysectomized at 10 A.M.–12 noon, by a method described elsewhere (13). Eleven mice were each injected with 25 I.U. PMS at 9 A.M. on the second day after the operation, a further eleven at 7 P.M., and the remainder at similar times on the third day. Uterine weights were recorded 22 hours after the injection. The hypophyseal fossa of each mouse after fixation in formalin was examined using a binocular microscope. The mice in which pituitary remnants were observed were not included in the analysis.

In each of the four groups (2 times of day×2 days) there were nine mice in which the pituitary had been completely removed. The analysis of variance (Table 6, Appendix) shows that there were no significant differences between responses at the different times of injection.

(b) Groups of 12 mice, half of which were ovariectomized, were injected with 2 μg. or 20 μg. oestradiol in water and killed 24 hours later. They were injected at 8–15 A.M. or at 4 P.M. the second day after weaning. Ovariectomy was performed one day after weaning. Body weights were 10–13 gm. at time of killing. The analysis of variance presented in Table 6 shows that there was no diurnal difference in the mean response in the ovariectomized mice or the normals. There was however, a significantly greater mean response in the ovariectomized mice. It is possible that the anesthesia caused liver damage which resulted in an enhancement of the effect of oestradiol through less efficient detoxication.

(c) Experiment V (b) was repeated without ovariectomy but the mice were killed at 16 hours. This was done in case there was an early diurnal difference which might have been eliminated at 24 hours. The mice were injected immediately after weaning. The results are not presented in detail since there was again no significant diurnal difference in the uterine response.

In both normal and ovariectomized animals the response of the uterus to injected oestrogen was thus independent of the time of injection.

Experiment VI

Since it seemed that the factor responsible for the diurnal variation on uterine and ovarian response was of pituitary origin it was thought likely that an extract of pituitary tissue might abolish the observed diurnal difference. It was also thought advisable to determine whether large doses of

PMS and HCG would affect the response. In addition an extract of postmenopausal women's urine was tested.

Five groups of each eight mice were injected at either 9 A.M. or 7 P.M. in either of two successive days. They were weaned 24 hours before injection and killed 21 hours after the injection. The injections at each time consisted of: HCG—0.3 I.U. (uterine weights) and 30.0 I.U. (uterine and ovarian weights); PMS—as for HCG; sheep anterior pituitary extract, fraction II, AP—3 mg. (uterine weights). Body weights were recorded at death.

The low level of HCG and PMS was given as a control; the results of the analysis of variance (Table 7, Appendix) show that there was a significant difference between A.M. and P.M. injections each day and, unlike Experiment I, there was no significant day-to-day difference in response. The dose of AP was approximately equivalent to each of the low levels of HCG and PMS. There were no significant differences between times of day or between days. The analysis of the data of the high levels of PMS and HCG is also shown in this table; there were no significant time-to-time differences for either uterine or ovarian weight. There was however a significantly greater mean uterine and ovarian response with PMS.

In another experiment the above results were confirmed, using an 0.5 *M* buffered saline extract of sheep pituitary glands. The preparation of postmenopausal women's urine (HMG) gave similar results to AP.

DISCUSSION

Mice injected with moderate doses of PMS and HCG in the evening clearly give greater uterine and ovarian responses 24 hours later than do mice treated similarly but injected in the morning. The phenomenon is exhibited equally well with both PMS and HCG. In addition, the evening injection causes an earlier beginning of ovarian and uterine growth and also results in significantly greater ovarian, but not uterine, weights at 48 hours.

An important problem in biological studies is the variability which exists between test subjects. The introduction of appropriate statistical measures will often bring such variation under the control of the operator. Nevertheless such measures may add greatly to the complexity of experiments which are conducted over a period because time-to-time variation is normally unpredictable and occasionally large. It is therefore of considerable importance that time-to-time variation in response to gonadotropin has been shown to have a diurnal component.

There are several mechanisms that might be responsible for the diurnal variation in response to PMS and HCG. The results of Experiments V (b) and (c) seem to eliminate the possibility that there is a diurnal variation in uterine responsiveness, and the findings in hypophysectomized

animals (Experiment V (a)) and the absence of a diurnal difference with pituitary extracts suggest that the phenomenon is not a manifestation of an intrinsic daily rhythm of sensitivity in the ovary, but that the fluctuations in ovarian and uterine responses find their origin in the pituitary gland. The results obtained with hypophysectomized animals are by no means clear cut however, for the sensitivity of the ovary to PMS in such animals is much lower than in intact animals (13). Nevertheless it is to be expected from a number of other studies (2, 6, 7, 8, 10) that such a diurnal rhythm would have a neuro-humoral mechanism involving the hypothalamus and the pituitary gland.

The ovaries and uteri of immature mice begin to exhibit increases in weight between 15 and 20 hours after the injection of gonadotropin (5) (and present data, Experiment IV). However the delay between injection of gonadotropin and the beginning of uterine and ovarian growth has been found to show significant variation with time of day, being longer when injection is given in the morning. Once it has begun, the rate of increase in weight of both organs does not depend on time of day. The finding that the increased delay after the morning injection is not abolished until the dosage levels of HCG and PMS are 20 or more times greater than that needed to produce a maximal uterine weight response, is of considerable significance.

It seems to indicate that there is a diurnal fluctuation in the output of a pituitary factor that is qualitatively different from the gonadotropin(s) assayed by the ovarian or uterine weight tests (FSH-like and LH-like gonadotropins). A diurnal variation in response of the uterus was not observed when doses of sheep anterior pituitary and human post-menopausal gonadotrophin which were approximately equivalent to 1 I.U. HCG or PMS as judged by the uterine response, were used, hence the diurnally fluctuating factor presumably cannot be the gonadotropin (14) measured by the uterine weight response. Since HCG and PMS appear to have brought about similar response trends to low and high levels and since PMS is rich in a gonadotropin biologically similar to FSH, it seems that the diurnally fluctuating factor cannot be FSH-like either. The results may therefore indicate the presence of a pituitary factor different from FSH and LH, which is necessary for steroid production or ovarian growth. In the evening the pituitary gland presumably produces more of this factor. Whether it is a gonadotropin however is open to question. At least it does not appear to be necessary for uterine response to oestrogen for not only was there no diurnal variation in response to oestradiol (Experiment V b and c) but hypophysectomy does not decrease the sensitivity of the uterus to oestradiol (Lamond, unpublished observations).

Another explanation of the present findings is suggested by the recent results of Lamond and Emmens (13) who showed that responses to the placental gonadotropins HCG and PMS are apparently limited by en-

dogenous gonadotropin production. If the animal's own pituitary gland responds to the injection of HCG or PMS then it may be that there is a diurnal variation in the rapidity of this response. A direct action of the placental gonadotropins on the neuro-hypophyseal axis has not, however, been demonstrated but may well repay further investigation. Alternatively the endogenous gonadotropins may not act on the ovary in a manner different from PMS and HCG but the amount produced may be considerably in excess of the effective amount of exogenous gonadotropin.

The time of day when the gonadotropin was injected had no influence on the uterine weight 48 hours later (i.e. at a time when uterine growth had ceased) but there was a difference at 48 hours in ovarian weights. The reason for the divergence between these two criteria of ovarian response is not apparent.

The results of Experiment III call for special mention. It is apparent that mice 14–17 days old are less sensitive to gonadotropin than mice 3–4 days older. Similar findings have been reported by Ortiz (15) for the rat and hamster. In the mice the lower sensitivity was particularly evident in the ovarian weight responses, which were not significantly greater than the controls. In the slightly older and bigger mice, not only was there a response in both uteri and ovaries, but there was also a period of increased ovarian responsiveness over the 12 hours from midday to midnight. In mice two weeks older and an estimated two weeks before puberty, this period was considerably shorter, being confined approximately to 2 P.M.–6 P.M. Hence in the strain of mice used in these experiments there seems to have been a gradual development of an overall responsiveness to HCG and PMS, and this has a diurnal component which becomes more closely confined to the mid-afternoon as the mice approach puberty. Pfeiffer (16) postulated that the sex type of the pituitary gland is not genetically determined but that its differentiation is dependent on the type of gonad present. It is possible that this differentiation is simply the development of a diurnal rhythm.

Acknowledgment

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REFERENCES

1. Austin, C. R. and A. W. H. Braden: *Aust. J. Biol. Sci.* **7**: 179. 1954.
2. Braden, A. W. H.: *J. Exp. Biol.* **34**: 177. 1957.
3. Browman, L. G.: *J. Exp. Biol.* **75**: 375. 1937.
4. Claringbold, P. J. and D. R. Lamond: *J. Endocrinol.* **16**: 86. 1957.
5. Emmens, C. W., P. J. Claringbold and D. R. Lamond: *Nature* **180**: 38. 1957.

6. Everett, J. W. and C. H. Sawyer: *Endocrinology* **45**: 581. 1949.
7. Everett, J. W. and C. H. Sawyer: *Endocrinology* **47**: 198. 1950.
8. Harris, G. W.: *Neural Control of the Pituitary Gland.* London: Edward Arnold Ltd. 1955.
9. Hemmingsen, A. N. and N. P. Krarup: *K. danske Vidensk. Selsk. Biol. Medd.* **13**: 5. 1937.
10. Hill, M. and A. S. Parkes: *Proc. Roy. Soc. B.* **113**: 537. 1933.
11. Lamond, D. R.: *Aust. vet. J.* **33**: 127. 1957.
12. Lamond, D. R.: *J. Endocrinol.* **17**: 216. 1958.
13. Lamond, D. R. and Emmens, C. W.: *J. Endocrinol.* (in press.) 1959.
14. Lamond, D. R. and P. J. Claringbold: *J. Endocrinol.* **16**: 298. 1958.
15. Ortiz, E.: *Physiol. Zool.* **20**: 45. 1947.
16. Pfeiffer, C. A.: *Amer. J. Anat.* **58**: 195. 1936.
17. Snell, G. D., E. Fekete, K. P. Hummel and L. W. Law: *Anat. Rec.* **76**: 39. 1940.

APPENDIX

Table 2. Analyses of variance of the data obtained in experiment 1

Mean squares headed (i) are comparisons between fresh preparations only and (ii) are comparisons between stored and fresh preparations injected on Day 3. The non-significant interactions in this and following experiments are pooled to form a residual term, which is not used in the estimate of error.

Source of variation	D.F.	Mean square			
		Uterine weight		Ovarian weight	
		(i)	(ii)	(i)	(ii)
Preparations (P)	1	3203***	1629***	22.88***	15.58***
Doses (D)	1	1613***	1272***	4.88***	3.66***
Time of day (T)	1	780**	557**	9.86***	16.47***
Replications (R)					
Linear	1	714**		1.65*	
Quadratic	1	6		1.62*	
Stored v Fresh (S)	1		447*		0.06
Interactions					
P×D	1	224	525**	7.40**	3.92**
P×D×T	1	326*		2.00*	
P×S	1		447*		0.11
Remainder	16	70		0.44	
	9		52		0.37
Error	127	71.3		0.37	

*** $P<0.001$.
** $0.001<P<0.01$.
* $0.01<P<0.05$.

TABLE 3. ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT II

Source of variation	D.F.	Mean square			
		Uterine weight		Ovarian weight	
		At 24 hr.	At 48 hr.	At 24 hr.	At 48 hr.
Preparations (P)	1	1134***	2592***	11.77***	55.56***
Doses (D)	1	640**	6864***	3.91**	28.20***
Time of day	1	706**	14	8.01***	19.84***
Interactions					
P×D	1		384*		5.48**
Remainder	4	55		0.78	
	3		53		0.32
Error	31	78.9	88.7	0.44	0.69

TABLE 4. ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT III

Source of variation	D.F.	Mean square		
		(a)	(b)	(c)
Time of day				
Linear	1	183	0	96
Quadratic	1	10	876***	433
Cubic	1	56	26	245
Quartic	1	17	1	1026**
Quintic	1	38	0	
Doses	1	2208***	70	
Interaction	5	59	30	
Error	47	71.6	42.3	
	29			133.7

TABLE 5. ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT IV

Source of variation	D.F.	Mean square		
		Uterine weights		Ovarian weights
		(a)	(b)	(a)
Time of day	1	465**	305**	2.40*
Time of killing				
Linear	1	7156***	1740***	24.22***
Quadratic	1	147	4	0.11
Cubic	1			0.10
Doses	1	184	99	
Interaction	7	31	63	
	3			0.33
Error	47	47		
	35		38	
	31			0.32

TABLE 6. ANALYSES OF VARIANCE OF THE RESULTS OF EXPERIMENTS V (a) AND V (b)

	Source of Variation	D.F.	Mean square
V (a)	Day 1 v Day 2	1	54
	A.M. V P.M.	1	22
	Interaction	1	4
	Error	31	37.6
V (b)	Normal v ovariectomized	1	999***
	A.M. V P.M.	1	1
	Between doses	1	2338***
	Interaction	4	40
	Error	39	43.1

TABLE 7. ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT VI

Source of variation	D.F.	Mean square			
		Uterine weights			Ovarian weights
		Low levels HCG, PMS	High levels HCG, PMS	AP	High levels HCG, PMS
Preparations	1	56	1156***		10.32***
Day 1 v day 2	1	225	0	145	0.88
A.M. V P.M.	1	1541***	56	45	0.70
Interaction	4	9	94		0.73
	1			18	
Error	55	89.5	63.8		0.35
	27			63.2	

A COMPARISON OF THE EFFECTS OF *IN VIVO* AND *IN VITRO* THYROID HORMONES UPON THE OXYGEN CONSUMPTION OF RAT KIDNEY SLICES[1]

ROGER L. GREIF AND JEAN MORONEY

Department of Physiology, Cornell University Medical College, New York City

ABSTRACT

Immediately after removal of one kidney from thyroidectomized rats a large amount of thyroxine or triiodothyronine was injected intravenously. Slices made from the remaining kidney, removed 10 minutes later, had QO_2 levels significantly higher than control values after storage at 4° C for 48 hours in alanine buffer. After the injection of labeled thyroxine the I^{131} content of the injected kidney corresponded to .03 μg. thyroxine per mg. wet weight. Diiodotyrosine, injected under similar conditions, did not affect the QO_2. Thyroxine added to slices from both control and injected kidneys was also effective in preserving QO_2.

MATERIALS with thyroid hormone activity are effective *in vitro* if added to ruptured cells (1), to their separated components (2, 3), or to ascites tumor tissue (4). Thibault (5) and Barker (6) have studied the effect of added thyronine derivatives upon the oxygen consumption of slices made from the kidneys of thyroidectomized rats. The slices were stored at 4° C between daily measurements of oxygen consumption (QO_2) at 37° C, and under these conditions the metabolically active thyronine derivatives delayed the expected decline in QO_2 with time to a striking degree.

The present experiments were designed to determine whether a similar delayed decline in QO_2 with time is present if the thyroid hormones reach the kidney via the circulatory system and the amount of thyroid hormone that is effective under these *in vivo* conditions.

METHODS

Male albino rats from the Charles River Breeding Laboratories were surgically thyroidectomized when body weight was 50 to 100 gm., at least two months before the experiment. They received Purina laboratory chow and tap water *ad libitum*. At the time of the experiment the animals were anaesthetized with ether, and through a mid-abdominal incision the right renal artery and vein were clamped with a hemostat. The right kidney was then removed and cooled on ice. 1 mg. of the material being tested, dissolved in dilute NaOH, was injected into the inferior vena cava in a volume of 0.5 cc.,

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and 10 minutes later the other kidney was removed. In a moist chamber two slices were made from the lateral aspects of each kidney with a Stadie-Riggs microtome (four slices per kidney), and in order to diminish error due to variable amounts of cortex and medulla, half of an inner and of an outer slice were weighed together and placed in each of two Warburg vessels. The buffer used throughout contained .12*M* NaCl, .003*M* KCl, .0012*M* $MgSO_4$, .017*M* DL-alanine (pH 7.8), and .01*M* glucose, final pH 7.2. The gas phase was oxygen, temperature 37° C, shaking speed 120 strokes per minute. QO_2 is based upon the first 30 minutes of observation, although the vessels were kept at 37° C for approximately 70 minutes daily. Between oxygen consumption measurements they were covered with paraffin film and stored at 4° C. When labeled thyroxine was used, radioactivity was measured by placing slices in tubes of cold buffer in a well-type scintillation counter prior to transferring them to Warburg vessels. DL-thyroxine was obtained from Nutritional Biochemicals Corp., 3,5,3′ L-triiodothyronine from Dr. A. E. Heming of the Smith, Kline, and French Corp., 3,5, diiodo-L-tyrosine from Eastman Kodak Co., and L-thyroxine labeled with I^{131} from Abbott Laboratories, Oak Ridge.

RESULTS

The table summarizes, with statistical evaluation, the QO_2 results measured at successive 24 hour intervals following the removal of the tissue. It can be seen that highly significant changes are produced by injection of materials with known metabolic activity. In the case of DL-thyroxine, the changes are seen after 48 hours, but they appear in 24 hours when 3,5,3′ L-triiodothyronine is used, and are absent in tissues taken from animals previously injected with diiodo-L-tyrosine. The same data are included in the figures as black bars. Slices from each kidney were also placed in contact with L-thyroxine, 10 μg. per cc. of buffer. In the figures the QO_2 values so obtained are shown by white bars. It can be seen in Figure 1 that after 48 hours in the presence of added thyroxine the QO_2 of the tissue remains high, and that this result is not affected by the prior injection of diiodo-L-tyrosine. Figures 2 and 3 show that a further increase can be produced by the *in vitro* exposure to thyroxine, even though the animal has previously been injected with a metabolically active hormone preparation. The increases in QO_2 from *in vitro* contact with thyroid hormone after 48 and 72 hours are all significant with a P value of less than .001.

Ten minutes after the injection of DL-thyroxine to which a small amount of I^{131} labelled L-thyroxine had been added, the tissue I^{131} level corresponded to .03 ± .004 (S.E.) μg. thyroxine per mg. wet weight (4 measurements on each of 9 animals).

DISCUSSION

The method of Barker (6) represents one of the few demonstrations of an *in vitro* effect of materials with thyroid hormone activity upon excised tissue containing whole cells. The complex changes which occur in tissue permeability following excision (7) might be thought to favor the entrance of hormone into the cell, and indeed, in a recent note (8) Barker and Etling have shown that added thyroxine is rapidly taken up by excised kid-

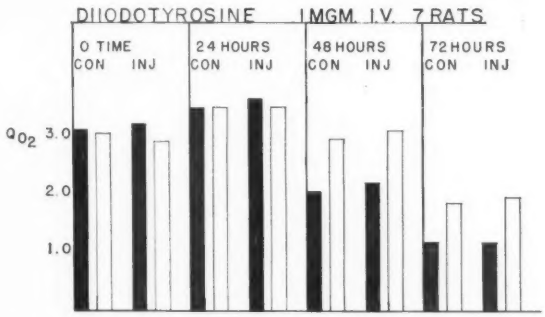

FIG. 1. Results of daily measurement of QO_2 following injection of diiodotyrosine. The black bars show the mean QO_2 for slices from control and injected kidneys suspended in buffer alone, the white bars show the mean QO_2 for slices from the same kidneys suspended in buffer containing 10 micrograms L-thyroxine per ml.

QO_2 OF SLICES MADE FROM CONTROL AND INJECTED KIDNEYS
1 MG. OF EACH COMPOUND INJECTED INTRAVENOUSLY

Time in hours	DL-thyroxine (16 rats)			L-triiodothyronine (10)			diiodotyrosine (7)		
	Con.	Inject.	P	Con.	Inject.	P	Con.	Inject.	P
0	3.04 ± .10*	2.90 ± .09	> .1	3.26 ± .11	3.20 ± .10	> .1	3.16 ± .08	3.25 ± .09	> .1
24	3.47 ± .10	3.41 ± .09	> .1	3.36 ± .07	3.60 ± .09	< .05	3.52 ± .12	3.68 ± .08	> .1
48	1.96 ± .07	2.35 ± .05	< .001	1.97 ± .03	2.32 ± .06	< .001	2.09 ± .11	2.24 ± .08	> .1
72	1.08 ± .04	1.08 ± .05	—	1.04 ± .07	1.22 ± .06	> .05	1.21 ± .09	1.20 ± .08	> .1

* Standard Error.

ney tissue. The present experiments, in which the hormones reach the tissue via the vascular system, demonstrate that the swelling of cells following excision need not precede contact with thyroid hormones for a demonstrable effect in preserving oxygen consumption, but add no information on the question of later tissue swelling as a consequence of contact with these compounds.

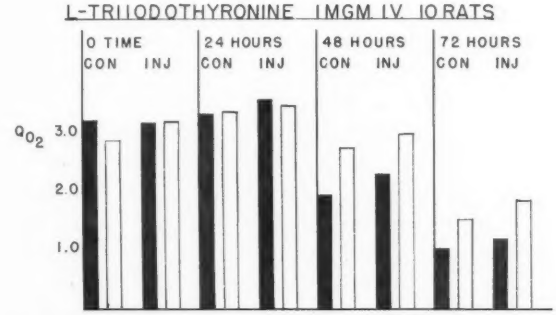

FIG. 2. Results of daily measurement of QO_2 following injection of 3,5,3′ L-triiodothyronine.

Legend as in Figure 1.

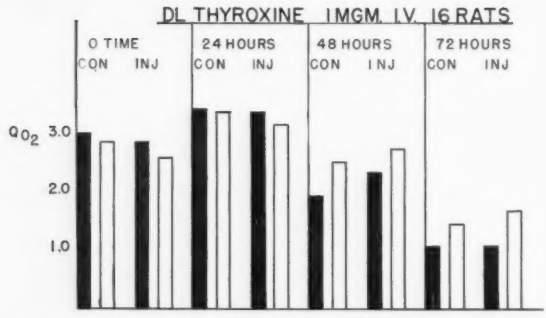

FIG. 3. Results of daily measurement of QO_2 following injection of DL-thyroxine. Legend as in Figure 1.

Since the effects of thyroid hormones reaching the cells via the vascular system and *in vitro* are additive, it seems probable that .03 μg. of thyroxine per mg. of tissue is insufficient under the present conditions to produce a maximum tissue response. This impression has been confirmed in a few experiments by noting a larger response after the injection of larger amounts of hormone.

Each flask in the present studies contained an average of 75 mg. of tissue in 3 cc. of buffer. Assuming that thyroxine added *in vitro* is all rapidly bound by kidney tissue (8), the experiments with injected hormone correspond to about 2 μg. of added thyroxine per vessel. This is close to the minimum amount reported to be active *in vitro* (9). Although experimental conditions are strikingly different, it is of interest to note that this concentration of thyroxine, $10^{-6}M$, has been shown to uncouple oxidation from phosphorylation in isolated mitochondria *in vitro* (10) but is 100 times the concentration required for the *in vitro* production of mitochondrial swelling (11).

In every experiment it can be seen that the QO_2 of kidney slices increases during the first 24 hours after excision. This phenomenon, which has also been observed by Barker (9), appears to be somewhat more striking in the presence of added or injected thyroid hormones than in their absence. A similar increase is seen following the injection of diiodotyrosine, however, which makes the relationship between this finding and thyroid hormone action questionable. The initial lowered QO_2 may be the result of impaired diffusion from compression of tissue during slicing, though we have not performed experiments to check this point.

In the present experiments there is a more rapid effect observed after the injection of triiodothyronine than after a similar amount of DL-thyroxine. On the basis of the rapid onset of metabolic activity following the injection of triiodothyronine (12), and the isolation of this compound from thyroid tissue after injection of I^{131} (13) it has been suggested that triiodothy-

ronine is a step closer to the form of the thyroid hormone which acts upon the cell. It should be pointed out, however, that the present experiments could well be explained on the basis of the rapid penetration and high tissue hormone concentration found after the injection of triiodothyronine as contrasted to thyroxine (14). The many possible variations in the present technique remain to be explored.

REFERENCES

1. Gemmill, C. L.: *Am. J. Physiol.* **167:** 349. 1951.
2. Hoch, F. L. and F. Lipmann: *Proc. Natl. Acad. Sci. U.S.* **40:** 909. 1954.
3. Lardy, H. A. and G. Feldott: *Ann. N. Y. Acad. Sci.* **54:** 631. 1951.
4. Heimberg, M. and J. H. Park: *Endocrinology* **57:** 756. 1955.
5. Thibault, O.: *C. R. Soc. de Biol.* (Paris) **149:** 877. 1955.
6. Barker, S. B.: *Endocrinology* **59:** 719. 1956.
7. Davies, R. E.: In "Active Transport and Secretion" Symposium of the Society for Experimental Biology VIII: Cambridge: 453. 1954.
8. Barker, S. B. and N. Etling: *Fed. Proc.* **17:** 31. 1958.
9. Barker, S. B.: *Ciba Foundation Colloquia on Endocrinology* **10:** 253. 1957.
10. Lehninger, A. L.: In "Enzymes: Units of Biological Structure and Function" (Ed.) Gaebler, O. H., Academic Press Inc. New York. p. 217. 1956.
11. Lehninger, A. L. and B. L. Ray: *Science* **125:** 748. 1957.
12. Asper, S. P., Jr., H. A. Selenkow and C. A. Plamondon: *Bull. Johns Hopkins Hosp.* **93:** 164. 1953.
13. Gross, J. and R. Pitt-Rivers: *Biochem. J.* **53:** 645. 1953.
14. Larson, F. and E. C. Albright: *Endocrinology* **63:** 183. 1958.

INVESTIGATION OF THE RELEASE OF ACTH[1]

H. W. RUMSFELD, JR. AND JOHN C. PORTER

Departments of Biochemistry and Physiology, The University of Texas, Southwestern Medical School, Dallas, Texas

ABSTRACT

The ACTH-releasing activity of portal vessel plasma, as estimated by adrenal ascorbic acid depletion in the steroid-depressed rat, was present in protein fraction III_0. The reduction in adrenal ascorbic acid concentration following injection of III_0 was not attributable to the presence of ACTH in this protein fraction since III_0 was inactive in the hypophysectomized rat. The remaining protein fractions had no significant effect on adrenal ascorbic acid concentration. The activity attributable to III_0 was lost during dialysis when the protein fraction was dissolved in a buffer solution containing 15.2% ethanol. This suggested either destruction of the active component or dissociation of a dialyzable component from the carrier protein.

Fraction III_0 from carotid artery plasma was inactive regardless of whether the blood was collected rapidly, slowly, or allowed to remain mixed with macerated skeletal muscle for two hours at 0° C.

The ACTH-releasing activity of portal plasma III_0 was not attributable to contamination with nor-epinephrine, epinephrine, histamine, or serotonin. Similarly, injection of NaCl solutions of different concentrations were ineffective.

THE primary cause of variation in the rate of ACTH discharge from the anterior pituitary in response to an endless array of environmental changes remains an enigma, and it is to be emphasized that the circumstances which decrease the rate of ACTH discharge are no less important in homeostasis than are those circumstances which increase ACTH release. Admittedly, it is difficult to ascribe specificity to a particular stimulus for the latter; however, determination of the effective site of action of an inhibitor of ACTH release is no less difficult. The results of some investigations suggest the involvement of a neurohumoral agent, "corticotrophin-releasing factor—CRF" (1), but a discordant view regarding the uniqueness of such a substance is not without significance. Such divergent views exist not only because of possible lack of quantity and purity of CRF but also because of lack of proven specificity and defined limits of applicability for the assay system used in estimating CRF activity. The difficulties inherent in the assay of CRF have been considered in a recent review (2). Nonethe-

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less, previous reports which suggested that blood collecting in the sella area following hypophysectomy (3, 4) and blood draining the brain (5, 6) accelerated the release of ACTH prompted further consideration of this problem. Preliminary reports of part of this work have been previously presented (7, 8).

MATERIALS AND METHODS

Plasma used in this investigation was obtained from blood of mongrel dogs of both sexes. Blood for experimental purposes was collected by aspirating that which accumulated in the sella area following removal of the pituitary, the source of this blood being in part the hypophyseal portal vessels. The procedure employed here was the same as that previously described (3). The plasma was stored at −20° C until used. The procedure followed in fractionation of the plasma proteins and the terminology used in referring to the different fractions was identical to that used by Cohn *et al.* (9). Dialysis was carried out with stirring at 4° C for approximately 2 hours utilizing Visking dialysis tubing,[2] size 24/32 with a specified average pore radius of 24 Å. In all cases the sample was dried by lyophilization before use in assay, and the dried sample was dissolved in 4 cc. of distilled water for injection.

Male albino rats were used in the assays. The hypophysectomized rat was prepared by the parapharyngeal approach and was used in the adrenal ascorbic acid depletion test 20 to 24 hours after hypophysectomy (10). The hydrocortisone-inhibited intact rat was prepared by injecting intraperitoneally 6 mg. of hydrocortisone acetate in saline per 100 gm. body weight 4 hours before use. Such an animal does not release ACTH in response to unilateral adrenalectomy (3). All solutions were administered through the tail vein in two injections of 2 cc. each. The first injection was given immediately after removal of the left adrenal, and the second was given 30 minutes later. The right adrenal was removed 90 minutes after the first injection. The first adrenal was removed under ether anesthesia; the second adrenal was removed under nembutal anesthesia. In each instance the total amount of lyophilized plasma or plasma fraction injected was equivalent to 10 cc. of original plasma.

RESULTS

The intravenous administration of lyophilized portal vessel plasma to hydrocortisone-inhibited intact rats resulted in significant reduction in adrenal ascorbic acid concentration ($P < 0.01$). Following partitioning of the portal plasma proteins into fractions IV+V+VI, I+III_3, II, $III_{1,2}$ and III_0, only fraction III_0 caused a significant reduction in adrenal ascorbic acid concentration ($P < 0.01$) (Table 1).

It has been reported (4) and confirmed in this investigation that the ACTH-releasing activity of portal vessel plasma was not affected by dialysis against 0.9% NaCl solution. However, extension of this observation to fraction III_0 must be restricted. The solution used to dissolve III_0 contained 0.6 moles glycine and 2.62 moles ethanol and was buffered with glycinate and phosphate at pH 6.8–6.9 (9). When this solution containing III_0 was lyophilized to remove the ethanol prior to dialysis against 0.9% NaCl solution, the change in adrenal ascorbic acid following injection of

[2] Visking Corporation, Chicago, Illinois.

TABLE 1. EFFECT OF PORTAL PLASMA AND PLASMA FRACTIONS ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-TREATED RATS

Number of rats	Weight of rats (gm.)	Ascorbic acid concentration (left adrenal at 0 min.) (mg./100 gm.) mean	Final treatment (two injections i.v.—2 cc. per injection)	Ascorbic acid concentration (right adrenal at 90 min.) (mg./100 gm.) mean	Change in adrenal ascorbic acid concentration (mg./100 gm.)
24	323 ± 11.6*	345	*0 min.*—lyophilized portal plasma equivalent to 5 cc. *30 min.*—lyophilized portal plasma equivalent to 5 cc.	261	−84 ± 6.3
12	294 ± 21.6	340	*0 min.*—fraction IV, V, VI equivalent to 5 cc. portal plasma *30 min.*—fraction IV, V, VI equivalent to 5 cc. portal plasma	334	− 6 ± 6.7
12	291 ± 12.4	333	*0 min.*—fraction I+III_2 equivalent to 5 cc. portal plasma *30 min.*—fraction I+III_2 equivalent to 5 cc. portal plasma	341	+ 8 ± 5.9
15	295 ± 14.6	349	*0 min.*—fraction II equivalent to 5 cc. portal plasma *30 min.*—fraction II equivalent to 5 cc. portal plasma	360	+11 ± 4.4
2	232 ± 13.0	370	*0 min.*—fraction $III_{1,2}$ equivalent to 5 cc. portal plasma *30 min.*—fraction $III_{1,2}$ equivalent to 5 cc. portal plasma	370	0 ± 3.5
59	300 ± 7.9	342	*0 min.*—fraction III_0 equivalent to 5 cc. portal plasma *30 min.*—fraction III_0 equivalent to 5 cc. portal plasma	316	−26 ± 5.4**

* Mean and standard error.
** This value includes the results from all assays regardless of treatment of III_0. An explanation of the discrepancy between this value and that for lyophilized plasma is suggested by the data presented in Table 2.

III_0 was -55 ± 9.7 mg. per 100 gm. adrenal weight for 18 determinations. However, when this same solution containing III_0 was dialyzed against 0.9% NaCl solution without prior lyophilization, the change in adrenal ascorbic acid following injection of III_0 was $+1 \pm 5.1$ mg. per 100 gm. adrenal weight for 18 determinations. The difference in these two means was statistically significant ($P < 0.01$) (Table 2).

When III_0 from portal plasma was assayed in the 24-hour hypophysectomized rat and the hydrocortisone-treated rat, the changes in adrenal ascorbic acid concentration were $+6 \pm 6.2$ mg. per 100 gm. adrenal weight for 7 determinations and -55 mg. ± 24.1 per 100 gm. adrenal weight for 6 determinations respectively. The difference in these two means was significant ($P = 0.03$) (Table 2). Fraction III_0 used here was from a common pool which was dried by lyophilization prior to dialysis.

Plasma protein fraction III_0 was prepared from (a) carotid artery blood which was permitted to flow rapidly during collection, (b) carotid artery blood which was allowed to drip into an iced beaker during a 3 hour collection period, and (c) a mixture of 200 cc. of carotid artery blood and 10 gm. of macerated skeletal muscle. This mixture remained in an ice bath for one hour before separation of the plasma. The mean change in adrenal

ascorbic acid for 7 determinations in each group was -3 ± 4.2, $+3 \pm 3.1$, and $+10 \pm 2.7$ mg. per 100 gm. adrenal weight respectively.

As it was difficult to maintain constant osmolarity of the solutions injected during these experiments, NaCl solutions of different concentrations were given intravenously to the steroid-inhibited rat in an attempt to assess the effect of solutions of varying osmolar concentrations on the release of ACTH. After injection of 4 cc. in two 2 cc. injections of 0.45, 0.90, 2.25, 3.15 and 4.05 per cent NaCl solutions, the change in adrenal ascorbic acid was $+9 \pm 2.0$, $+12 \pm 0.7$, $+1 \pm 3.1$, $+13 \pm 3.0$ and $+7 \pm 6.1$ mg. per

TABLE 2. EFFECT OF DIALYSIS OF PLASMA FRACTION III_0 ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-TREATED RATS

Number of rats	Weight of rats (gm.)	Ascorbic acid concentration (left adrenal at 0 min.) (mg./100 gm.) mean	Final treatment (two injections i.v.—2 cc. per injection)	Ascorbic acid concentration (right adrenal at 90 min.) (mg./100 gm.) mean	Change in adrenal ascorbic acid concentration (mg./100 gm.)
18	316 ± 12.6*	340	*0 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis without prior lyophilization) *90 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis without prior lyophilization)	341	$+1 \pm 5.1$
18	290 ± 12.7	339	*0 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis with prior lyophilization) *90 min.*—fraction III_0 equivalent to 5 cc. portal plasma (dialysis with prior lyophilization)	284	-55 ± 9.7

* Mean and standard error.

100 gm. adrenal weight respectively. These observations suggested that variation in osmolarity of injected solutions within these limits had no effect on the release of ACTH in these steroid-treated rats. Following intravenous administration of 8 μg. of nor-epinephrine, 8 μg. of epinephrine and 6 mg. of histamine hydrochloride, the change in adrenal ascorbic acid concentration was $+22 \pm 8.0$, -1 ± 2.2 and $+7 \pm 5.6$ mg. per 100 gm. adrenal weight respectively. The change in adrenal ascorbic acid in the steroid-treated rat injected with a total of 40 μg. of serotonin[3] was -1 ± 3.3 mg. per 100 gm. adrenal weight, of 80 μg. of serotonin was 0 ± 1.0 mg., of 160 μg. of serotonin was $+2 \pm 1.6$, and of 320 μg. of serotonin was -2 ± 10.0.

DISCUSSION

These results supported the suggestion (3, 4) that blood that drained into the sella area of the hypophysectomized dog stimulated the release of ACTH in the hydrocortisone-treated rat as estimated by adrenal ascorbic acid depletion. Following fractionation of the portal plasma proteins,

[3] Injected as serotonin·creatinine·H_2SO_4·H_2O complex. The authors express appreciation for the generous gift of serotonin from Vismara Terapeutici. This was obtained through the California Foundation for Biochemical Research, Los Angeles.

ACTH-releasing activity was present only in fraction III_0. No change in adrenal ascorbic acid was observed following injection of an equal amount of III_0 in the hypophysectomized rat, suggesting that the source of ACTH released here as a result of injection of III_0 was the pituitary. As the likelihood existed that the ACTH-releasing activity in portal plasma was attributable to the prolonged standing of the portal vessel blood in an ice bath during the collection period, carotid artery blood was allowed to drip into a cold container during a two-hour collection period in an attempt to control this variable. Also, to account for possible tissue contamination macerated skeletal muscle was mixed with carotid artery blood and the mixture allowed to stand in an ice bath for one hour. The injection of plasma fraction III_0 from each of these samples of carotid artery blood did not cause reduction in adrenal ascorbic acid of the steroid-depressed rat.

The loss in ACTH-releasing activity of portal plasma III_0 following dialysis when this fraction was still dissolved in a solution containing 15.2% (v/v) ethanol at pH 6.8–6.9 suggested destruction of the active component or the dissociation of a dialyzable substance from the carrier protein. Lyophilization prior to dialysis prevented such loss of activity. Since the net result of lyophilization here was the removal of ethanol and water, the latter of necessity being partly replaced to make dialysis possible, it would seem that the presence of ethanol under these conditions promoted destruction or dissociation of the active substance.

It is interesting to speculate regarding the possible identity of the active component in III_0 and DΔ, a preparation having no pressor activity (11, 12). McCann, using rats with hypothalamic lesions, was unable to separate pressor activity and ACTH-releasing activity of Pitressin (13). However, interpretation of observations regarding the role of the antidiuretic hormone as well as other substances in the regulation of ACTH release from the pituitary is complicated by the observations that Pitressin potentiated the effect of ACTH on cortical steroid production in the dog (14) and that Pitressin and arginine vasopressin caused adrenal ascorbic acid depletion in the 'decapitated' rat (15). Fraction III_0 from portal plasma is without effect on adrenal ascorbic acid in the hypophysectomized rat under the conditions of the present experiments. This does not exclude the possibility that greater quantities would be effective. Since nor-epinephrine, epinephrine, histamine, serotonin and sodium chloride solutions of varying concentrations did not significantly decrease the concentration of adrenal ascorbic acid in the hydrocortisone-inhibited intact rat, it was concluded that the ACTH-releasing activity of III_0 was not due to contamination with these substances or to different osmolarities of the injected solutions. It will be of interest to see if the results of future investigations justify the restriction of corticotrophin-releasing activity to a single substance or show it to be a property of several substances. The results of this investigation, however, are in accord with the view that a neurohumoral agent is

transported to the anterior pituitary through the portal vessels and that this substance increases the rate of release of ACTH from the pituitary.

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The authors wish to express their thanks to Miss Elizabeth Parsons, Mrs. Julia Hallford and Mr. Tom Sullens for excellent technical assistance during the course of this investigation.

REFERENCES

1. Saffran, M., A. V. Schally and B. G. Benfey: *Endocrinology* **57**: 439. 1955.
2. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **20**: 243. 1958.
3. Porter, J. C. and J. C. Jones: *Endocrinology* **58**: 62. 1956.
4. Porter, J. C. and H. W. Rumsfeld, Jr.: *Endocrinology* **58**: 359. 1956.
5. Schapiro, S., J. Marmorston and H. Sobel: *Proc. Soc. Exper. Biol. and Med.* **91**: 382. 1956.
6. Schapiro, S., J. Marmorston and H. Sobel: *Am. J. Physiol.* **192**: 58. 1958.
7. Porter, J. C. and H. W. Rumsfeld, Jr.: *Fed. Proc.* **15**: 146. 1956.
8. Porter, J. C. and H. W. Rumsfeld, Jr.: *Communications of the XXth International Physiological Congress*: 737. 1956.
9. Cohn, E. J., F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma: *J. Am. Chem. Soc.* **72**: 465. 1950.
10. Sayers, M. A., G. Sayers and L. A. Woodbury: *Endocrinology* **42**: 379. 1948.
11. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Housholder: *Endocrinology* **60**: 488. 1957.
12. Clayton, G. W., W. R. Bell and R. Guillemin: *Proc. Soc. Exper. Biol. and Med.* **96**: 777. 1957.
13. McCann, S. M.: *Endocrinology* **60**: 664. 1957.
14. Hume, D. M. and D. H. Nelson: *Endocrine Soc.* (abst.) **98**: 1957.
15. Royce, P. C. and G. Sayers: *Proc. Soc. Exper. Biol. and Med.* **98**: 70. 1958.

FURTHER STUDY OF AN ACTH-RELEASING PROTEIN FROM HYPOPHYSEAL PORTAL VESSEL PLASMA[1]

JOHN C. PORTER AND H. W. RUMSFELD, JR.

Departments of Physiology and Biochemistry, The University of Texas, Southwestern Medical School, Dallas, Texas

ABSTRACT

Fraction III_0 obtained from hypophyseal portal vessel plasma by the Cohn fractionation procedure possessed all of the ACTH-releasing activity of portal plasma. This fraction consisted of three major protein components with isoelectric points at pH 4.4, pH 5.1 and pH 6.6. Separation of the components of this fraction by starch column electrophoresis yielded an active peak with a mobility in veronal buffer (pH 8.6; $\mu = 0.1$) of $-1.91 \pm .41$ cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^{-5}$.

The activity associated with the proteins of fraction III_0 was destroyed by incubation with trypsin and pepsin and by refluxing with 0.1 N NaOH. It was not destroyed by two hours of refluxing with 0.1 N HCl.

Preliminary results indicated that the active substance could be dialyzed away from the proteins in distilled water if the lyophilized portal plasma was dissolved in a Cohn Buffer at pH 6.9. This would suggest that this plasma protein serves in a transport role for an ACTH-releasing substance in portal vessel blood.

THE ACTH-releasing activity of hypophyseal portal vessel plasma (1, 2) was found in one protein fraction (3). This investigation deals with further purification of this protein fraction, electrophoretic characterization of its components, and the determination of some of its properties which may help to elucidate its role in the release of ACTH. Preliminary results of part of this work have been previously reported (4, 5).

MATERIALS AND METHODS

Plasma used in this investigation was obtained from blood of mongrel dogs of both sexes. Blood for experimental purposes was collected by aspirating that which accumulated in the sella area following removal of the pituitary, the source of this blood being in part the hypophyseal portal vessels. The procedure employed here was the same as that previously described (1). The plasma was stored at $-20°$ C until used. It was fractionated by the low temperature alcohol fractionation method of Cohn *et al.* (6), and the terminology used in identifying the different fractions was the same as that used by Cohn. The use of the hydrocortisone-inhibited intact rat in estimating ACTH-

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releasing activity was the same as that previously described (1), and depletion of adrenal ascorbic acid was used as the criterion of ACTH release (7).

Electrophoretic mobility of the III_0 fractions was determined by moving boundary electrophoresis of 1% protein solutions. Protein concentration was measured by a biuret method (8). The solutions were dialyzed against the following buffers of 0.1 ionic strength (μ): acetate at pH 3.6 and pH 4.5, cacodylate at pH 5.6, phosphate at pH 6.8 and veronal at pH 8.6; and electrophoresis was carried out in a Perkin-Elmer model 38A electrophoresis apparatus. Buffer resistance was measured at 0° C with a standard conductivity cell and an impedance bridge.

The III_0 proteins were further separated by starch zone electrophoresis (9) using acetate buffer at pH 3.6 ($\mu = 0.1$), 150–160 volts and 15–20 milliamperes for periods ranging from 22 to 48 hours. Analysis for protein in aliquots from 1 cm. segments of the column was by the Folin-Lowry procedure (10). Segments corresponding to a peak were pooled, dialyzed, lyophilized and assayed for ACTH-releasing activity. The mobility of pooled active peaks from the starch columns was determined by moving boundary electrophoresis in veronal buffer at pH 8.6 ($\mu = 0.1$).

All dialyses in this investigation were carried out with stirring at 4° C utilizing Visking dialysis tubing[2], size 24/32 with a specified average pore radius of 24 Å.

For tryptic and peptic digestions of III_0, aliquots equivalent to 10 cc. of original plasma and containing 51 mg. of protein were dialyzed for two hours against two changes of 1.5 liters of 0.9% NaCl solution and then lyophilized. One aliquot was then assayed again for activity. Another aliquot was dissolved in 50 cc. of 0.5% Na_2CO_3 solution, adjusted to pH 8.3 with HCl and mixed with 5 mg. of Difco trypsin. A third aliquot was dissolved in 50 cc. of 0.1 N HCl and mixed with 5 mg. of Difco pepsin. Incubation was carried out with shaking in a constant temperature bath at 37° C for 3 hours. The samples were then removed, heated in a boiling water bath for 8 minutes, cooled in ice, filtered and adjusted to pH 7.0 with HCl and NaOH respectively. The samples were then lyophilized for assay.

Additional aliquots of fraction III_0 or portal plasma were refluxed in 0.1 N NaOH for 1 hour and in 0.1 N HCl for 1 and 2 hours. The samples were then neutralized, lyophilized and assayed unless otherwise stated in the results section.

The solution for the dialysis of portal plasma at pH 5.0 was made with 0.1 M ammonium formate buffer. This buffer was later removed by sublimation (11).

RESULTS

It has been demonstrated that the ACTH-releasing activity of portal vessel plasma was attributable to protein fraction III_0 (3). Fraction III_0 from portal plasma was further separated by moving boundary electrophoresis. Three components were evident at five different pH values, and their mobilities were determined (Table 1). Graphic representation of the electrophoretic mobilities as a function of pH showed the isoelectric points of the three components to be at pH 4.4, pH 5.1 and pH 6.6 (Fig. 1). Figure 2 illustrates an electrophoretic pattern obtained by moving boundary electrophoresis of fraction III_0 in acetate buffer at pH 3.6 ($\mu = 0.1$). A similar pattern was obtained when the proteins of III_0 were separated by starch zone electrophoresis when this same acetate buffer was used. The ACTH-releasing activity migrated in that part of the distribution which

[2] Visking Corporation, Chicago, Illinois.

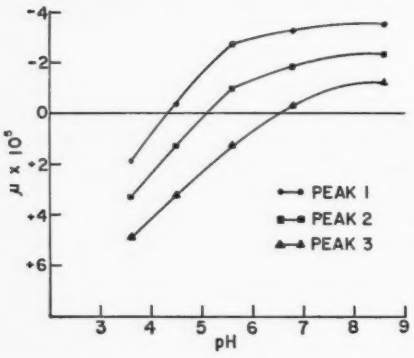

FIG. 1. Electrophoretic mobility (μ) of fraction III_0 components as a function of pH. The isoelectric points occur at the following pH values: 4.4, 5.1 and 6.6. The numbering of the peaks here is identical to that used in Table 1.

corresponded to the second of three protein peaks. The active portion is indicated by the shaded area in Figure 3 which also corresponded to the second peak observed by moving boundary electrophoresis (Table 1). The latter had a mobility of $-2.47 \pm .09$ cm.^{2} volt^{-1} sec.$^{-1} \times 10^{-5}$ in veronal buffer at pH 8.6 ($\mu = 0.1$) (Table 1) and an isoelectric point at pH 5.1 (Fig. 1). However, when the active protein peaks from several starch electrophoretic separations were pooled and then measured by moving boundary electrophoresis at pH 8.6 ($\mu = 0.1$) in veronal buffer, a single peak with a mobility of $-1.91 \pm .41$ was found. This would be compatible with the view that the active protein corresponded to a slow component of peak 2 or a fast component of peak 3 (Table 1). ACTH-releasing activity was not re-

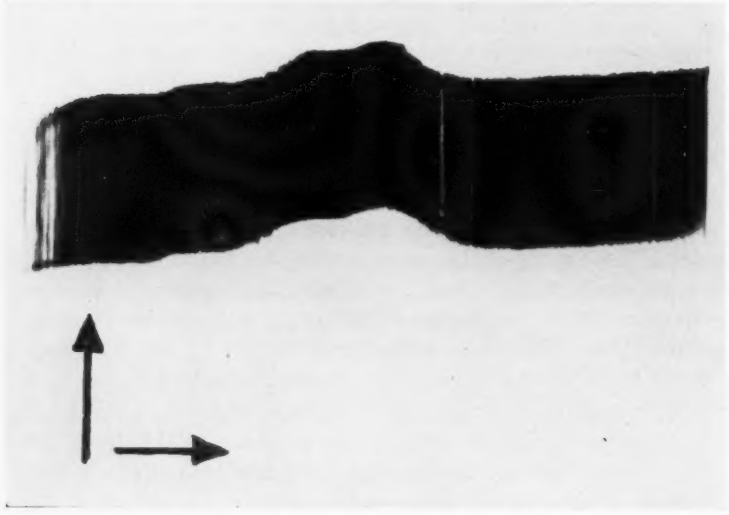

FIG. 2. Moving boundary electrophoresis of fraction III_0 in acetate buffer at pH 3.6 and 0.1 ionic strength.

TABLE 1. ELECTROPHORETIC MOBILITY OF THE PROTEIN COMPONENTS OF FRACTION III_0 FROM HYPOPHYSEAL PORTAL VESSEL PLASMA

Buffer	pH	Ionic strength (μ)	Mobility*		
			Peak 1	Peak 2	Peak 3
Veronal	8.6	0.1	$-3.43 \pm .13$**	$-2.47 \pm .09$	$-1.25 \pm .30$
Phosphate	6.8	0.1	$-3.23 \pm .06$	$-1.88 \pm .22$	-0.28
Cacodylate	5.6	0.1	$-2.71 \pm .02$	$-0.98 \pm .17$	$+1.29 \pm .09$
Acetate	4.5	0.1	$-0.37 \pm .06$	$+1.27 \pm .14$	$+3.23 \pm .13$
Acetate	3.6	0.1	$+1.89$	$+3.31 \pm .47$	$+4.91 \pm .43$

* $cm.^2\ volt^{-1}\ sec.^{-1} \times 10^{-5}$.
** Mean and standard deviation.

covered from the starch column in every case following electrophoretic separation of III_0. However, in those separations in which activity was recovered the quantity of protein in the active peak varied from 6 to 36% of the total amount of protein. The median percentage was 15.6%. The pattern chosen for illustration in Figure 3 was representative; the active peak had 12% of the total protein on the column.

The activity was destroyed by incubation with trypsin or pepsin for 3 hours and by refluxing for 1 hour with 0.1 N NaOH. Conversely, the activity of III_0 was not destroyed by refluxing with 0.1 N HCl for periods up to 2 hours duration (Table 2).

Dialysis of portal plasma against 0.1 N HCl for 25 hours or against ammonium formate at pH 5 for 24 hours did not affect the ACTH-releasing ac-

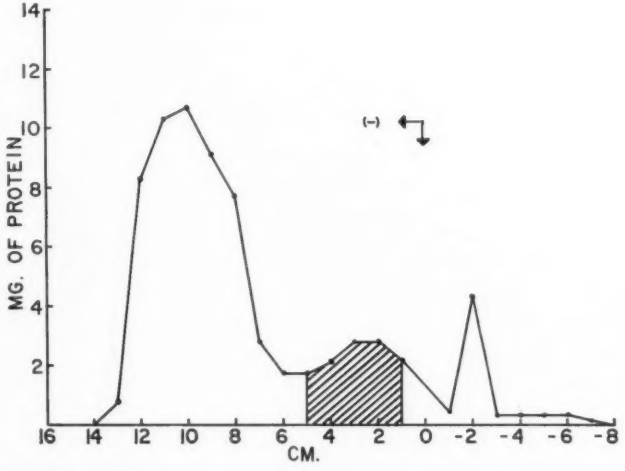

FIG. 3. Separation of the components of fraction III_0 by starch column electrophoresis in acetate buffer at pH 3.6 and 0.1 ionic strength. The column was run for $22\frac{3}{4}$ hours at 160 volts and 18 milliamperes at a temperature of 4° C. The ACTH-releasing activity was present in that portion of the III_0 proteins illustrated by the shaded area of this pattern.

TABLE 2. EFFECT OF TRYPSIN, PEPSIN AND REFLUXING WITH NaOH AND HCl ON ACTH-RELEASING ACTIVITY OF FRACTION III_0 FROM PORTAL VESSEL PLASMA

Number of rats	Sample	Treatment	Change in adrenal ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
2	III_0 5 cc. equivalent*	None	-51 ± 12.0**
4	III_0 10 cc. equivalent	None	-95 ± 21.1
1	III_0 10 cc. equivalent	3 hr. incubation with trypsin at pH 8.3 at 37° C	+ 5
1	III_0 10 cc. equivalent	3 hr. incubation with pepsin in 0.1 N HCl at 37° C	+ 1
1	III_0 10 cc. equivalent	Reflux 1 hr. with 25 cc. 0.1 N NaOH	+ 6
1	III_0 5 cc. equivalent	Reflux 1 hr. with 25 cc. 0.1 N HCl	−33
1	III_0 10 cc. equivalent	Reflux 2 hr. with 25 cc. 0.1 N HCl	−61

* 5 cc. and 10 cc. equivalent refers to that volume of fraction III_0 from the Cohn fractionation equivalent to 5 cc. and 10 cc. of original portal plasma, respectively. III_0 contains approximately 5 mg. protein per 1 cc. equivalent.
** Mean and standard error.

tivity; no activity was detected in the dialysate (Table 3). However, when lyophilized portal plasma was placed in the buffer solution used to dissolve fraction III_0 during plasma protein fractionation (3), activity was found outside the dialysis bag following dialysis against distilled water. Some activity was also found outside the bag when portal plasma was dialyzed for 24 hours against 15.2% (v/v) ethanol. An ethanol concentration of 15.2% (v/v) was identical with that in the buffer solution used to dissolve III_0 during plasma protein fractionation (6). When an ethanol-portal plasma mixture (78% ethanol by volume) was allowed to stand at −20° C for 48 hours, all of the ACTH-releasing activity remained with the protein precipitate. Following similar treatment of carotid artery plasma, there was no activity in either the precipitate or supernatant.

DISCUSSION

Following the observation that lyophilized hypophyseal portal plasma would elicit ACTH release in the steroid-treated rat, as measured by adrenal ascorbic acid depletion, it was found that the ACTH-releasing activity was not lost on dialysis. This activity was in protein fraction II+III (2); it was found later that the ACTH-releasing activity of portal plasma was present in a subfraction of III, *viz.* III_0 (3, 4, 5).

In the present study III_0 was observed to consist of three major proteins with isoelectric points at pH 4.4, pH 5.1 and pH 6.6. Assay of the proteins of fraction III_0 which had been separated by starch column electrophoresis showed that intermediate peak 2 (Table 1), or more likely a component of this peak, was associated with the active substance. Moving boundary electrophoresis in veronal buffer of combined active peaks from the starch column electrophoretic separations demonstrated a single component with a mobility, -1.91 ± 0.41 cm.^{2} volt.$^{-1}$ sec.$^{-1} \times 10^{-5}$, midway between that of

TABLE 3. STUDIES ON THE DISSOCIATION OF ACTH-RELEASING ACTIVITY FROM PORTAL PLASMA PROTEIN

Volume of portal plasma	Treatment	Change in adrenal ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)		
		Prior to treatment	After treatment	
			Inside dialysis bag	Outside dialysis bag
15 cc.	(a) sample adjusted to pH 1 (b) dialyzed for 25 hrs. against a total volume of 1 liter of 0.1 N HCl (c) dialyzate was changed 4 times	−112*	− 98	+ 4
15 cc.	(a) refluxed with 25 cc. of 0.1 N HCl for 1½ hrs. (b) dialyzed for 6 hrs. against a total volume of 1 liter distilled water (c) dialyzate was changed 3 times	− 98	− 99	—
15 cc.	(a) sample adjusted to pH 5.0 with formic acid (b) dialyzed for 24 hrs. against a total volume of 1 liter of ammonium formate buffer at pH 5.0 (c) dialyzate changed 4 times	−112*	−142	—
15 cc.	(a) sample adjusted to pH 6.9 with the buffer used to dissolve fraction III_0 during Cohn fractionation (b) dialyzed for 24½ hrs. against a total volume of 890 cc. of distilled water. (c) dialyzate was changed 4 times	−112*	− 36 − 89	−42
10 cc.	(a) — (b) dialyzed for 24 hrs. against a total volume of 750 cc. of 15.2% (v/v) ethanol (c) dialyzate was changed 4 times	—	− 57	−25

* A 10 cc. aliquot was assayed prior to treatment.

peaks 2 and 3 (Table 1). The isoelectric point of this protein would be between pH 5.1 and pH 6.6. The amount of protein in peak 2 varied from 6 to 36% of the total protein. These observations suggested that the active constituent might be associated with a smaller amount of protein than is usually found in this peak.

During the preparation of fraction III_0 for assay it was previously found that the order in which lyophilization and dialysis were carried out affected the activity of the resulting product (3). Dialysis prior to lyophilization resulted in loss of activity; this could be interpreted as destruction of the active substance or dissociation of the active material from the protein. In this regard it was of possible significance that an active protein component was not always recoverable following starch column electrophoretic separations. The lack of success in these instances, however, was by no means apparent by inspection of the resulting protein distribution.

Destruction of the activity of III_0 occurred during incubation with

trypsin and pepsin and during refluxing with 0.1 N NaOH. However, the active component was quite stable to refluxing in 0.1 N HCl. Following dialysis against distilled water for 24 hours, ACTH-releasing activity was present in the dialysate when lyophilized portal plasma was dissolved in the Cohn buffer used to fractionate III_0 (6).

This observation would support the postulate that the constituent in portal plasma responsible for ACTH release in the steroid-treated rat is dissociable from the plasma proteins. In view of these results dissociation rather than destruction of the active substance would appear to be the most likely cause for the loss of activity of III_0 previously reported (3). It is interesting to speculate that the protein component of III_0 described here functions in a transport role for a neurohumoral agent which increases the rate of release of ACTH.

Acknowledgment

The authors wish to express their thanks to Miss Elizabeth Parsons, Mrs. Julia Hallford and Mr. Tom Sullens for excellent technical assistance during the course of this investigation.

REFERENCES

1. Porter, J. C. and J. C. Jones: *Endocrinology* **58**: 62. 1956.
2. Porter, J. C. and H. W. Rumsfeld, Jr.: *Endocrinology* **58**: 359. 1956.
3. Rumsfeld, H. W., Jr. and J. C. Porter: *Endocrinology*. **64**: 942. 1959.
4. Porter, J. C. and H. W. Rumsfeld, Jr.: *Fed. Proc.* **15**: 146. 1956.
5. Porter, J. C. and H. W. Rumsfeld, Jr.: *Communications of the XXth International Physiological Congress*: 737. 1956.
6. Cohn, E. J., F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma: *J. Am. Chem. Soc.* **72**: 465. 1950.
7. Sayers, M. A., G. Sayers and L. A. Woodbury: *Endocrinology* **42**: 379. 1948.
8. Johnson, B. C. and A. M. Swanson: *J. Dairy Science* **35**: 823. 1952.
9. Kunkel, H. G. and R. J. Slater: *Proc. Soc. Exper. Biol. and Med.* **80**: 42. 1952.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall: *J. Biol. Chem.* **193**: 265. 1951.
11. Hirs, C. H. W., S. Moore and W. H. Stein: *J. Biol. Chem.* **195**: 669. 1952.

THE ACTION OF Δ^1 HYDROCORTISONE ON THE ANTIDIURETIC AND ADRENOCORTICOTROPIC RESPONSES TO NOXIOUS STIMULI[1]

DAVID DE WIED[2] AND I. ARTHUR MIRSKY

with the technical assistance of

LOUISA LINN AND EDWARD KRIMMER

Department of Clinical Science, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Rats treated with large doses of Δ^1 hydrocortisone do not exhibit a decrease in the adrenal ascorbic acid concentration after exposure to painful stimuli, injection with histamine or injection with nicotine. Such animals do respond to ACTH. Hydration results in a greater diuresis in Δ^1 hydrocortisone-treated than in untreated rats. Exposure to noxious stimuli produces the same antidiuretic response in treated and untreated rats if such measures are taken in 20 or more hours after the injection of the corticosteroid. The minimal dosage of Pitressin intraperitoneally necessary to produce a significant decrease in the concentration of the adrenal ascorbic acid is more than 10 times greater in the hydrocortisone-treated than in the untreated rats. A significant decrease in adrenal ascorbic acid is produced in the treated animals by the intravenous injection of approximately twice the dosage required intraperitoneally by the untreated rat. Increasing the intravenous dosage produces no further depletion of ascorbic acid in the adrenals of treated animals. Identical adrenal cortical responses are obtained in the hydrocortisone-treated rats with similar intravenous doses of Pitressin, arginine-vasopressin and lysine-vasopressin.

The data are interpreted to indicate that whereas the treatment with Prednisolone does not affect the responsiveness of the hypothalamus, it decreases markedly the responsiveness of the adenohypophysis and neurohypophysis of animals exposed to noxious stimuli.

EXPOSURE of animal or man to noxious stimuli results in an antidiuretic response (1, 2) and a marked increase in the antidiuretic (2, 3, 4) and adrenocorticotropic (5, 6) activities of the blood. The close association between the antidiuretic and adrenocorticotropic responses to noxious stimuli led to the hypothesis that the antidiuretic hormone (ADH), vasopressin, may be responsible for the activation of the adenohypophysis and the release of adrenocorticotropic hormone (ACTH) into the circulation (2, 7). In support of this hypothesis is the rapidity with

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[2] Present address: University of Groningen, Groningen, Holland.

which ADH is released into the circulation of animals exposed to noxious stimuli (1, 2), the rapidity with which the hypothalamus is depleted of its neurosecretory material and the associated vasopressin activity (7), the relative efficacy with which exogenous vasopressin induces a release of ACTH into the circulation (8, 9, 10, 11, 12), and the inhibition of the discharge of ACTH into the circulation of animals with lesions which prevent the release of vasopressin from the hypothalamus (13). In accord also are the observations that some pharmacological agents which inhibit the pituitary-adrenal response to a variety of stimuli do not appear to impair significantly the response to Pitressin (9, 10, 11, 14).

If the secretion of ACTH is dependent upon the secretion of vasopressin, then an agent which blocks the release of ACTH might operate by inhibiting the discharge of vasopressin. Accordingly, it became pertinent to investigate the influence of treatment with such an agent on the discharge of vasopressin into the circulation. Since hydrocortisone appears to be an efficient inhibitor of the mechanism responsible for the release of adrenocorticotropin, the effect of treatment with Δ^1 hydrocortisone on the antidiuretic and adrenal ascorbic acid responses to noxious stimuli were determined.

MATERIALS AND METHODS

Male albino rats of the Carworth strain, weighing from 120 to 220 gm. were used after a preliminary fast of approximately 16 hours during which time the animals were permitted access to water. During the experiments the rats were kept in individual metabolic cages. The animals were given 5 ml. of 0.2% sodium chloride per 100 gm. body weight by stomach tube and one hour later the gavage was repeated. At 20 minute intervals after the second gavage, the volume of urine excreted was measured and all rates of excretion were expressed as ml. per 100 gm. of body weight. The animals were handled and prodded just prior to each measurement of urine volume in order to insure the emptying of their bladders. The noxious stimuli were applied at 20 minutes after the second gavage. The animals that were treated with the steroid were given a subcutaneous injection of 10 mg. Δ^1 hydrocortisone (Prednisolone®)[3] in 0.5 ml. 70% alcohol per 100 gm. body weight at 20 to 24 hours before the first gavage.

Three types of noxious stimuli were used in this study: (1) painful stimuli produced by a two-minute period of mild electric shocks (50 volts, 60,000 ohms) to the feet through a grid which replaced the floor of the metabolic cage, (2) the intraperitoneal injection of 1 mg. histamine dihydrochloride, and (3) the intraperitoneal injection of 0.1 mg. nicotine tartrate per 100 gm. body weight. The rats that served as controls for the histamine and nicotine-treated groups received an injection of an equivalent volume of normal saline. The creatinine concentration of the urine was measured by the technique described by Peters (15).

The antidiuretic activity of the plasma was assayed by a procedure which was essentially that of Jeffers *et al.* (16). In accord with the observations reported by Dettelbach (17), the rats were prepared for the assay by gavaging them three times weekly with 10 ml. of a 0.2% saline solution during at least two weeks. The day of the assay, 2 or 3 animals were selected and given 5 ml. of a 2% solution of ethanol in 0.2% saline

[3] We are indebted to Dr. H. F. Hailman of the Upjohn Company for generous supplies of Prednisolone.

at 37° C per 100 gm. body weight by stomach tube. One hour later, the same amount of a 10 to 12% solution of ethanol in 0.2% saline was administered. At the end of the next hour each animal was given the same volume of the 2% ethanol solution. The animal that excreted the largest volume of urine during this period was selected for the assay. The bladder of the animal was exposed by a midline suprapubic incision and a polyethylene catheter inserted and fixed in place. The load of water was kept constant at between 7 to 8% of the body weight by gavaging the rat every ten minutes with an amount of the 2% ethanol equivalent to that excreted in the previous 10 minutes. All injections were made by means of a siliconized 22-gauge needle which was inserted into one of the lateral tail veins and kept in place with ties and tape. Blood from decapitated rats was collected in plastic tubes containing approximately 5 units heparin per ml., the plasma separated rapidly and assayed within 30 minutes. A maximum amount of 0.1 ml. plasma per 100 gm. body weight was injected into the assay animal. The volume of urine excreted during the 10 minutes after an injection was expressed as the percentage of that excreted for the 10 minutes prior to the injection. A preparation of Pitressin[4] was used as a standard and the antidiuretic activity of the plasma was expressed in terms of mU. Pitressin per 100 ml.

The decrease in the ascorbic acid concentration of the adrenal glands was used as a gauge of corticotropin release. The rats were decapitated one hour after exposure to the noxious stimuli, the adrenal glands removed, dissected clean and the ascorbic acid content determined by the method of Roe and Kuether (18) as modified by Sayers *et al.* (19). Animals given injections of normal saline served as controls. All animals were adapted for the assay by the daily intraperitoneal injection of 0.5 ml. saline for at least four consecutive days prior to the experiment.

RESULTS

Adrenal Ascorbic Acid Response

Twenty hours after the subcutaneous injection of Δ^1 hydrocortisone, 11 rats were exposed to the painful stimuli, 9 rats were given an intraperitoneal injection of histamine and a similar number of animals were given an injection of nicotine. Similar numbers of rats served as controls for each group and were injected intraperitoneally with saline. The data depicted in Figure 1 reveal that the dose of Δ^1 hydrocortisone used in this study completely prevented the reduction in the concentration of ascorbic acid which usually follows exposure to noxious stimuli. Whereas the concentrations (mean $\pm$S.E.) of the adrenal ascorbic acid of the animals exposed to pain, injected with histamine and injected with nicotine was 563 ± 21.6, 566 ± 29.9 and 543 ± 14.9 mg.%, those of the respective controls were 557 ± 20.5, 554 ± 30.1 and 519 ± 14.6 mg.%.

To exclude the possibility that the inhibition of the response to noxious stimuli was due to an impairment in the responsiveness of the adrenal cortex, the action of adrenocorticotropin was studied. A group of 16 rats was treated with Δ^1 hydrocortisone and 20 hours later, 8 animals were each given an intraperitoneal injection of 1 mU. adrenocorticotropin per 100 gm. of body weight and 8 were injected with saline. One hour later the adrenals

[4] We are indebted to Dr. D. A. McGinty of the Parke, Davis and Company for generous supplies of Pitressin.

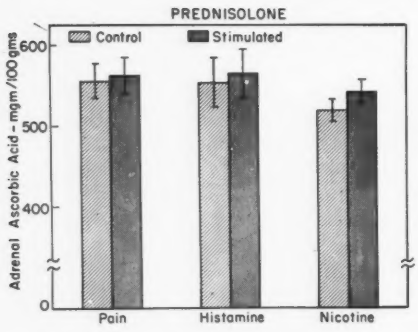

FIG. 1. Effect of Various Noxious Stimuli on the Concentration of Ascorbic Acid in Adrenals of Δ^1 Hydrocortisone-Treated Rats.

The bars depict the concentration of ascorbic acid (mean $\pm$ S.E.) in adrenals of animals exposed to the stimuli and of controls injected with saline, one hour before removal of the glands.

were removed and the ascorbic acid concentration determined. Whereas the adrenal ascorbic acid of the saline-injected rats was 474 ± 16.8 mg.%, that of the adrenocorticotropin-injected rats was 399 ± 17.8 mg. % ($P < 0.01$).

Antidiuretic Response

The rate of urine excretion (mean $\pm$ S.E.) by the saline-treated rats used as controls was fairly constant (Fig. 2). Exposure to the painful stimuli, however, resulted in a significant antidiuretic response. Whereas the rats exposed to the painful stimuli excreted 0.88 ± 0.09 ml. during the second twenty minute interval, their controls excreted 1.54 ± 0.08 ml. during the same interval ($P < 0.001$).

In accord with the observations of Gaunt *et al.* (20) who first demonstrated that corticosteroids promote the excretion of water, the administration of Δ^1 hydrocortisone resulted in an increased output of urine. Whereas rats treated with Prednisolone excreted 3.34 ± 0.08 ml., the saline-treated rats excreted 1.87 ± 0.14 ml. during the hour following the first gavage. Likewise, an increased rate of urine excretion occurred after the second gavage, especially during the first 40 minutes after the second

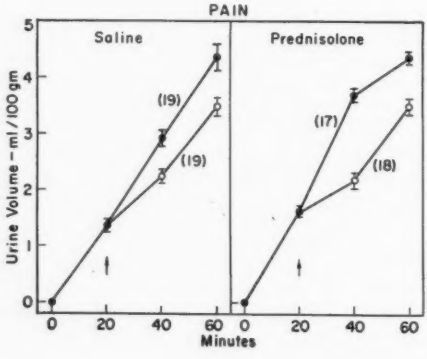

FIG. 2. Effect of Δ^1 Hydrocortisone on the Antidiuretic Response of Rats to Painful Stimuli.

The curves represent volume of urine excreted at each designated interval (mean $\pm$ S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.

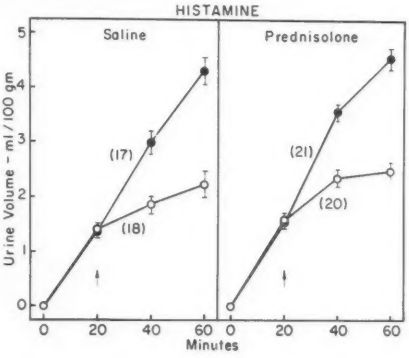

FIG. 3. Effect of Δ^1 Hydrocortisone on the Antidiuretic Response to Histamine.

The curves represent volume of urine excreted at each designated interval (mean $\pm$S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.

gavage (Fig. 2); the hydrocortisone-treated rats excreted 3.62 ± 0.13 ml. and the saline-treated group excreted 2.86 ± 0.13 ml. per 100 gm. body weight ($P < 0.01$). Although the rate of excretion by the Prednisolone-treated rats was not constant during the three 20-minute intervals following the second gavage, a significant antidiuretic response followed exposure to the painful stimuli. Thus, the rats exposed to the noxious stimuli excreted 0.55 ± 0.10 ml. during the 20-minute interval following the pain, while their controls excreted 2.09 ± 0.06 ml. ($P < 0.001$). The antidiuretic response of the Δ^1 hydrocortisone-treated group was even more pronounced than that of the saline-treated group.

A more intense antidiuretic response occurred after the administration of histamine and nicotine (Fig. 3 and 4). Saline-treated rats injected with histamine excreted 0.43 ± 0.12 ml. during the second, and 0.39 ± 0.11 ml. during the third 20-minute interval, while their respective controls excreted 1.64 ± 0.11 and 1.31 ± 0.11 ml. during the same intervals ($P < 0.001$). Prednisolone-treated rats behaved essentially the same way. Thus, the saline-injected rats excreted 2.02 ± 0.11 ml. and 0.98 ± 0.13 ml. during the two consecutive periods after the injection, while the histamine-treated rats excreted 0.78 ± 0.11 ml. and 0.15 ± 0.04 ml. respectively ($P < 0.001$).

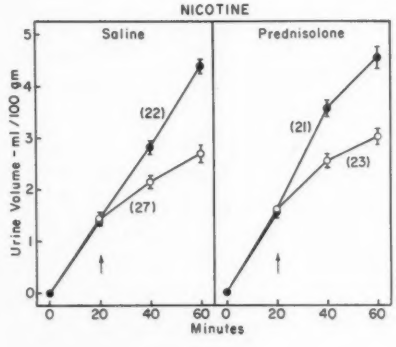

FIG. 4. Effect of Δ^1 Hydrocortisone on the Antidiuretic Response to Nicotine.

The curves represent volume of urine excreted at each designated interval (mean $\pm$S.E.) by saline-treated and hydrocortisone-treated rats. At the time designated by the arrow, one group of rats was exposed to the painful stimuli (○) while the other (●) was given an injection of saline. The numerals designate the number of animals in each group.

The normal rats given nicotine excreted 0.73 ± 0.17 ml. and their saline-injected controls excreted 1.44 ± 0.07 ml. during the first 20 minutes after injection. During the third 20-minute period after the gavage, the excretion of the nicotine-injected group amounted to 0.54 ± 0.08 ml. as compared to 1.56 ± 0.07 ml. excreted by the control animals ($P < 0.001$). In Δ^1 hydrocortisone-treated rats these values amounted to 0.94 ± 0.10 ml. as compared to 2.02 ± 0.11 ml. in the second 20-minute interval and 0.46

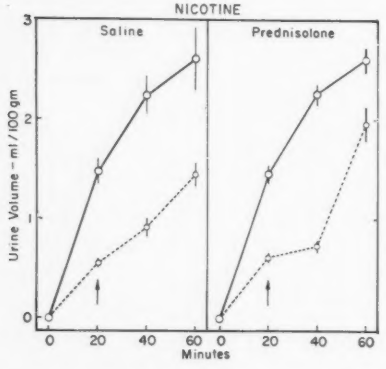

FIG. 5. Effect of Nicotine on Volume and Creatinine Concentration of Urine Excreted by Saline- and Δ^1 Hydrocortisone-Treated Rats.

Each curve depicts the volume of urine (mean $\pm$ S.E.) (○——○) and concentration of creatinine (mean $\pm$ S.E.) (○- - - -○) at designated intervals before and after the injection of nicotine (↑).

± 0.08 ml. as compared to 0.98 ± 0.13 ml. for their respective controls during the last 20-minute period ($P < 0.001$).

In order to establish that the antidiuretic response of Prednisolone-treated and untreated rats was due to an increase in the reabsorption of water rather than to a decrease in glomerular filtration, the creatinine concentration of the urine before and after the injection of nicotine was measured (Fig. 5). The creatinine concentration of the urine excreted by the untreated rats during the first 20-minute interval was 5.4 ± 0.4 mg.% before the injection of nicotine and rose to 9.1 ± 1.0 and 14.5 ± 1.2 mg.% during the following two 20-minute periods. In the Δ^1 hydrocortisone-treated rats the concentration of creatinine in the urine was 6.1 ± 0.7 mg.% before the injection, and 7.8 ± 0.5 mg.% and 19.6 ± 1.7mg.% during the second and third 20-minute intervals after the injection. In both groups the creatinine concentration of the urine excreted during the third 20-minute period was significantly higher than that found before the injection ($P < 0.001$).

The antidiuretic activity of the plasma was assayed in order to determine its relation to the antidiuretic response of rats as measured by the decrease in urine excretion. The activity of the plasma (mean $\pm$ S.E.) of normal rats was equivalent to 1.1 ± 0.33 mU. Pitressin per 100 ml. (Fig. 6). Thirty seconds after the rats were exposed to the painful stimuli the antidiuretic activity was equivalent to 3.9 ± 1.2 mU. per 100 ml. plasma. A more pronounced increase in the antidiuretic activity of the plasma

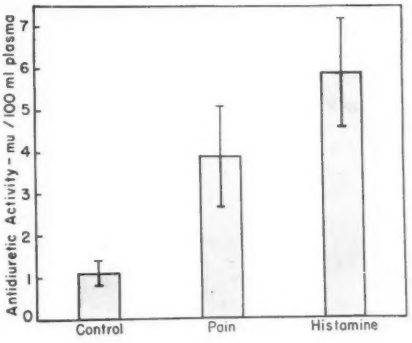

FIG. 6. Effect of Painful Stimuli and of Histamine on the Antidiuretic Activity of the Plasma.

Each bar represents the antidiuretic activity of the plasma of 16 rats (mean ± S.E.) expressed in terms of Pitressin (see text).

(5.9 ± 1.3 MU. per 100 ml.) was found in 10 minutes after the intraperitoneal injection of histamine ($P < 0.01$).

Response to Pitressin

The effect of the *intraperitoneal* injection of various doses of Pitressin on the concentration of adrenal ascorbic acid in saline and Δ^1 hydrocortisone-treated rats is detailed in Table 1 and illustrated in Figure 7. It is evident that a significant reduction in the adrenal ascorbic acid concentration of saline-treated rats followed the injection of 0.1 or more units of Pitressin per 100 gm. body weight. In accord with general experience the initial concentration of ascorbic acid in the adrenals of the rats treated

TABLE 1. EFFECT OF PITRESSIN ON THE ASCORBIC ACID CONCENTRATION OF THE ADRENAL GLANDS OF Δ^1 HYDROCORTISONE-TREATED AND UNTREATED RATS

The Pitressin was administered intraperitoneally (a) or intravenously (b) and the adrenals were removed one hour later for assay. The numerals in parentheses refer to the number of animals.

Pitressin units per 100 gm.	Adrenal ascorbic acid—mg. per 100 gm.		
	Controls	Prednisolone	
	a	a	b
0	394 ± 9.3 (26)	—	—
0.02	368 ± 14.5 (27)	—	—
0	393 ± 10.4 (23)	—	516 ± 22.7 (15)
0.1	312 ± 12.7 (22)***	—	490 ± 26.4 (15)
0	397 ± 11.7 (18)***	479 ± 17.7 (10)	521 ± 11.0 (16)**
0.2	273 ± 9.8 (19)	495 ± 20.0 (10)	451 ± 13.2 (16)
0		479 ± 17.7 (10)	477 ± 11.5 (15)**
0.5		488 ± 14.6 (10)	399 ± 12.5 (15)
0	414 ± 16.9 (10)***	431 ± 14.7 (15)*	505 ± 16.9 (9)*
1.25	233 ± 16.1 (10)	361 ± 24.2 (15)	422 ± 20.0 (10)

* Significant at 5% level.
** Significant at 1% level.
*** Significant at 0.1% level.

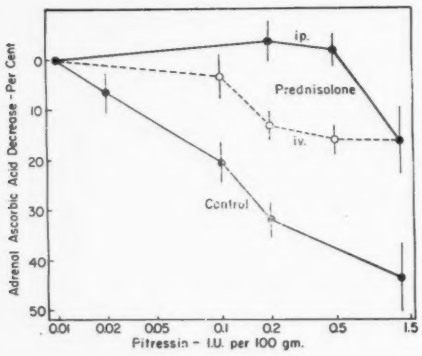

FIG. 7. Effect of Pitressin on the Adrenal Ascorbic Acid Δ^1 Hydrocortisone-Treated Rats.

The change in adrenal ascorbic acid concentration expressed as the per cent (mean $\pm$ S.E.) of values found in controls.

with Prednisolone was much higher than that found in untreated rats. Yet, the Δ^1 hydrocortisone-treated rats did not show a significant change in the adrenal ascorbic acid concentration until a dose of 1.25 units Pitressin per 100 gm. body weight was administered. Thus, more than ten times the dosage of Pitressin was necessary to produce the same adrenal ascorbic acid depletion in the Δ^1 hydrocortisone-treated rats as in the saline-treated controls.

The *intravenous* injection of 0.2 unit Pitressin per 100 gm. body weight was effective in reducing the concentration of ascorbic acid in the adrenals of the Prednisolone-treated rats. Increasing the dosage to as high as 1.25 U. per 100 gm., however, did not produce a further depletion of the ascorbic acid (Fig. 7).

That the adrenal ascorbic acid response to the intravenous injections to Pitressin is probably not due to some contaminant is suggested by the effects of purified arginine-vasopressin and synthetic lysine-vasopressin.[5] Three groups of 16 rats were treated with Δ^1 hydrocortisone and 20 hours later, half of each group was given an intravenous injection of 0.2 pressor unit of one of the antidiuretic compounds per 100 gm. of body weight while the other half of each group was injected with saline. One hour later the adrenals were removed and the ascorbic acid concentration determined. The data (mean $\pm$ S.E.) depicted in Figure 8 reveal that the three compounds produced essentially the same depletion in the adrenal ascorbic acid.

DISCUSSION

In accord with previous studies (2, 21), the experiments described herein reveal that exposure to various noxious stimuli such as pain, the injection of histamine and the injection of nicotine results in an antidiuretic response in normal rats. The concomitant increase in the concentration of creatinine in the urine indicates that the antidiuretic response is due to an

[5] We are indebted to Dr. Vincent du Vigneaud for generous supplies of compounds.

increase in the rate of water reabsorption by the renal tubules rather than to a decrease in glomerular filtration. The concomitant increase in the antidiuretic activity of the plasma suggests that the increased reabsorption of water by the renal tubules is due to the discharge of vasopressin into the circulation.

The antidiuretic response which occurs after exposure of an animal to a noxious stimulus may be due to the discharge of the antidiuretic hormone (ADH) directly from the hypothalamus into the general circulation and/or the discharge of ADH from the neurohypophysis (1, 2, 7, 21, 22, 23, 24, 25, 26). The essentially identical antidiuretic responses of the Δ^1 hydrocortisone-treated and saline-treated rats suggest that the steroid did not affect

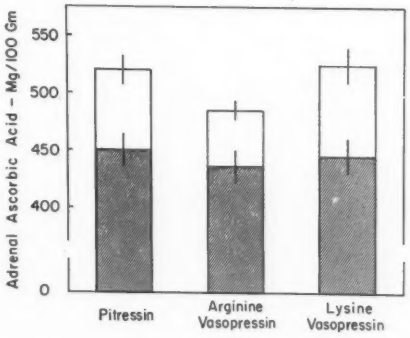

FIG. 8. Effect of Intravenous Injection of Pitressin, Arginine-Vasopressin and Lysine-Vasopressin on Adrenal Ascorbic Acid of Δ^1 Hydrocortisone-Treated Rats.

Each group consisted of 16 rats treated with hydrocortisone. Half of each group was injected with saline (open bars) and half with the designated compound (cross-hatched bars). One hour later the adrenals were removed and the ascorbic acid concentration (mean $\pm$ S.E.) determined.

the functional integrity of the hypothalamus. A block to the discharge of ADH from the neurohypophysis, however, is not precluded by these data. That Δ^1 hydrocortisone may induce an inhibition of the mechanism responsible for the discharge of ADH from the neurohypophysis without affecting that of the hypothalamus is suggested by the observations of Gaunt *et al.* (25). They found that the injection of formalin resulted in a marked reduction in the concentration of ADH in the hypothalamus of both normal and hydrocortisone-treated rats. The same insult resulted in a decrease in the concentration of ADH in the neurohypophysis of normal rats and an increase in that of the hydrocortisone-treated rats. Such an inhibition of the mechanisms responsible for the discharge of ADH from the neurohypophysis may account for the greater diuresis which occurs after hydration in animals treated with hydrocortisone than in controls.

The antidiuretic responses of the Δ^1 hydrocortisone-treated rats are not in complete agreement with the data reported recently by McCann *et al.* (27) who found that four hours after the intraperitoneal administration of 8 mg. hydrocortisone acetate per 100 gm. body weight, rats develop only about one half of the antidiuretic response observed in normal animals exposed to noxious stimuli. The discrepancy, however, is due to the fact that whereas McCann *et al.* performed their experiments in four hours

after the injection of the hydrocortisone, the data reported herein were collected in 20 to 24 hours after the administration of Prednisolone. Thus, when a group of 10 rats were hydrated and given nicotine four hours after the subcutaneous injection of Δ^1 hydrocortisone, they developed an antidiuretic response which was only 43 per cent of that exhibited by a group of 10 saline-treated controls. A similar decrease in the antidiuretic response occurred when rats were given nicotine 2 hours after the injection of the Δ^1 hydrocortisone. When the same groups were retested 25 hours after the initial subcutaneous injections, however, identical antidiuretic responses to nicotine were obtained in both the Δ^1 hydrocortisone and saline-treated groups.

Although McCann *et al.* (27) attribute the partial inhibition of the antidiuretic response to noxious stimuli to an inhibition of the hypothalamus, it is quite possible that other mechanisms may be involved. Thus, the immediate response may reflect an inhibition of the peripheral action of vasopressin such as was demonstrated by Gaunt *et al.* (20, 25). Likewise, the marked diuresis which occurs immediately after the injection of the corticosteroids may mask the antidiuretic response to noxious stimuli. It was in order to obviate the effects of these and other factors that the present studies were performed 20 to 24 hours after the injection of the Δ^1 hydrocortisone by which time there is no impairment in the antidiuretic response.

The decrease in the concentration of adrenal ascorbic acid which ordinarily follows exposure to noxious stimuli does not occur in the Δ^1 hydrocortisone-treated rats. Since the adrenal glands of animals treated with hydrocortisone respond to the administration of adrenocorticotropin, it is probably that the lack of response in noxious circumstances is due to an inhibition of the mechanisms responsible for the discharge of adrenocorticotropin into the circulation. Such an inhibition may be due to (a) a pharmacologically induced block in the hypothalamus similar to that which occurs with lesions in the median eminence (10, 11, 12, 13, 27, 28, 29) or (b) an inhibition of the production and/or release of adrenocorticotropin from the adenohypophysis.

That a decrease in the responsiveness of the mechanisms responsible for the release of corticotropin from the adenohypophysis can account for the effects of Δ^1 hydrocortisone is indicated by the difference in the responses of treated and untreated rats to different doses of Pitressin. Whereas a significant decrease in the concentration of the adrenal ascorbic acid of normal rats follows the *intraperitoneal* injection of 0.1 unit Pitressin per 100 gm. body weight, more than 10 times this dosage (1.25 U. per 100 gm. body weight) is required for a similar response in Δ^1 hydrocortisone-treated animals. Likewise, although the *intravenous* injection of 0.2 U. Pitressin per 100 gm. body weight does produce a significant decrease in the adrenal ascorbic acid of hydrocortisone-treated animals, increasing

the dosage to 1.25 U. per 100 gm. does not result in a further significant change. Such data support the probability that the absence of an adrenal cortical response to noxious stimuli in Δ^1 hydrocortisone-treated rats is due to a decrease in the responsiveness of the ACTH releasing mechanism rather than to an inhibition in the production of the hypothetical corticotropin releasing factor by the hypothalamus.

It would appear from the preceding that the corticosteroids act directly on the pituitary gland to reduce the responsiveness of the mechanisms responsible for the release of ACTH from the anterior lobe and ADH from the posterior lobe. This hypothesis is in accord with the studies of Rose and Nelson (30) on the suppressive effect of the injection of hydrocortisone directly into the fossa hypophysis on the release of ACTH. In accord also is the adrenal cortical atrophy that occurs in corticosteroid-treated dogs (28, 29) and rats (27) with the hypothalamic lesions that prevent the usual adrenal cortical responses to noxious stimuli. Accordingly, whereas the response of the normal animal to noxious stimuli results from the stimulation of both the hypothalamus and the hypophysis, the response of the Δ^1 hydrocortisone-treated animal may be due primarily to stimulation of hypothalamic mechanisms.

The nature of the neurohormonal agent (or agents) responsible for the activation of the release of ACTH from the adenohypophysis is unknown. As indicated at the outset, a good deal of evidence can be marshalled in support of the hypothesis that vasopressin or a closely related polypeptide is the specific corticotropin releasing agent. This hypothesis appears to be supported by the identical adrenal cortical responses of Δ^1 hydrocortisone-treated rats to the intravenous injections of similar doses of Pitressin, arginine-vasopressin and lysine-vasopressin. However, the evidence that the steroid produces a marked impairment in the responsiveness of the adenohypophysis makes it impossible to utilize the present study to either support or negate the postulate that vasopressin plays a prepotent role in the release of ACTH in the animal exposed to noxious stimuli.

REFERENCES

1. Verney, E. B.: *Proc. Roy. Soc. (London) B.* **135**: 25. 1947.
2. Mirsky, I. A., M. Stein and G. Paulisch: *Endocrinology* **54**: 491. 1954.
3. Ginsburg, M. and H. J. Heller: *Endocrinology* **9**: 274. 1953.
4. Robinson, K. W. and W. V. MacFarlane: *Austral. J. Biol. Sci.* **9**: 130. 1956.
5. Sydnor, K. L. and G. Sayers: *Endocrinology* **55**: 621. 1954.
6. Farrel, S. L. and S. M. McCann: *Endocrinology* **50**: 274. 1952.
7. Fraja, A. and L. Martini: *Arch. Int. Pharmacodyn.* **93**: 167. 1953.
8. Rothballer, A. B.: *Anat. Record.* **115**: 21. 1953.
9. Porter, J. C. and H. W. Rumsfeld: *Endocrinology* **58**: 359. 1956.
10. de Wied, D., P. R. Bouman and P. G. Smelik: *Endocrinology* **62**: 605. 1958.
11. McCann, S. M.: *Endocrinology* **60**: 664. 1957.
12. Smelik, P. G. and D. de Wied: Experientia. XIV: 17. 1958.
13. McCann, S. M. and J. R. Brobeck: *Proc. Soc. Exp. Biol. and Med.* **87**: 369. 1954.

14. Sevy, R. W., E. A. Ohler and A. Weiner: *Endocrinology* **61**: 45. 1957.
15. Peters, J. H.: *J. Biol. Chem.* **146**: 179. 1942.
16. Jeffers, W. A., M. A. Liverey and H. A. Austin: *Proc. Soc. Exp. Biol. and Med.* **50**: 184. 1942.
17. Dettelbach, H. R.: *Am. J. Physiol.* **192**: 379. 1958.
18. Roe, J. H. and C. A. Kuether: *J. Biol. Chem.* **147**: 399. 1943.
19. Sayers, M. A., G. Sayers and L. A. Woodbury: *Endocrinology* **42**: 379. 1948.
20. Gaunt, R., J. H. Birnie and W. J. Eversole: *Physiol. Rev.* **29**: 281. 1949.
21. Mirsky, I. A., G. Paulisch and M. Stein: *Endocrinology* **54**: 691. 1954.
22. Lloyd, C. W., E. Loewy, S. Pierog, K. Bradwick and R. Sostheim: *Proc. Soc Exp. Biol. and Med.* **85**: 333. 1954.
23. Keller, A. D.: *Endocrinology* **30**: 408. 1942.
24. Pickford, M.: *Lectures on the Scientific Basis of Medicine.* Volume IV: 1954–55.
25. Gaunt, R., C. W. Lloyd and J. J. Chart: *The Neurohypophysis.* H. Heller, Editor, Butterworths Scientific Publications, London. 1957.
26. Scharrer, E. and B. Scharrer: *Rec. Prog. in Horm. Res.* **10**: 183. 1954.
27. McCann, S. M., A. Fruit and B. D. Fulford: *Endocrinology* **63**: 29. 1958.
28. Ganong, W. F. and D. M. Hume: *Endocrinology* **55**: 474. 1954.
29. Ganong, W. F. and D. M. Hume: *Proc. Soc. Exp. Biol. and Med.* **88**: 528. 1955.
30. Rose, S. and J. F. Nelson: *Austral. J. Exper. Biol.* **34**: 77. 1956.

CARDIAC GLYCOGEN AND THE EFFECT OF GROWTH HORMONE AMONG INBRED STRAINS OF MICE[1]

JOHN B. LYON, JR.[2]

Department of Biochemistry, Division of Basic Health Sciences, Emory University, Georgia

ABSTRACT

In an effort to detect differences in the peripheral activity of growth hormone among C_{57}, A, C_3H, and I strain mice, the concentrations of cardiac glycogen were determined during the course of a 24-hour fasting period, and in three different age groups (young, 40–50 days; young-adult, 3–5 months; and old, 8–13 months). The concentrations and changes in cardiac glycogen were found to be characteristic of the age, and of the strain of mice. For example, in young-adult, but not young, mice of the I strain, cardiac glycogen actually decreased during a 24-hour fast. Some strain differences were found, but these were not consistent in all age groups. It was concluded that cardiac glycogen was subject to a variety of influences, which in turn varied with the age of the animal.

As found for the rat, exogenous growth hormone doubled the stores of cardiac glycogen but the hormone had to be administered in the morning to non-fasted mice. If it was administered in the afternoon, it was not effective. However, if insulin was given either before or with the growth hormone in the afternoon, an effect usually seen with administration of growth hormone in the morning could be duplicated. Insulin alone had no effect. This, and other evidence, suggests that insulin is required for the action of growth hormone on cardiac glycogen deposition.

STUDIES of the lipid (1), protein (2), and carbohydrate (3) metabolism of four highly inbred strains of mice have demonstrated that each strain possesses a distinctive metabolic pattern. In attempts to account for these metabolic patterns by hormonal differences, it was tentatively postulated that one of the strains, the I, has a high peripheral activity of insulin (3, 4, 5, 6). However, the possibility that the unusual carbohydrate metabolism in this strain was due to other hormonal influences could not be overlooked. Since the large stores of glycogen in the abdominal and gastrocnemius muscles of the I strain do not deplete as readily during a fast as the smaller stores in the musculature of the C_{57}, A or C_3H strains (3), the presence of larger amounts of a "carbohydrate sparing factor," such as growth hormone, in the I strain seemed possible. However, higher quantities of

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growth hormone in the other strains seemed equally possible, for the endogenous nitrogen excretion of the A strain is less (7), and the growth rate of the A or the C_3H strain is greater (2) than that of the I strain. One feasible experimental approach to this question was the determination of the stores of cardiac glycogen in these strains. Russell and her collaborators have demonstrated that the presence of growth hormone is necessary for the increase in cardiac glycogen which occurs during a fast (8, 9), and that increases in cardiac glycogen following the administration of growth hormone are directly related to the dose of the hormone (10). Moreover, the action of growth hormone on cardiac glycogen seems to be independent of insulin (8, 9).

METHODS

Animals. Mice of the A/FnLn, C_{57}BL/FnLn, I/FnLn and C_3H strains from our colony were used in these experiments. Groups of mice were housed in plastic cages and were maintained on a commercial stock ration (Purina Laboratory Chow). Except in the first group (Section A), all of the mice were males, 3–5 months of age. Food and water were supplied *ad libitum*, and during fasting periods, water was supplied *ad libitum*. The mice were anesthetized with pentobarbitol-sodium (Nembutal, Abbot), 0.1 mg. per gm. body weight.

Removal of the heart. In preliminary studies, the concentration of glycogen found in the mouse heart was considerably lower than that reported for the rat heart, although the concentrations of glycogen in the gastrocnemius or diaphragm muscles are similar in the two species (3, 9). Considering the greater metabolic and heart rate of the mouse, it was possible that glycogenolysis occurred to a greater extent in the process of removing the mouse heart. It has been shown that glycogenolysis occurs in the rat heart when the heart is beating in the opened chest (11). A simple technique was evolved for a rapid removal. The skin and fur over the rib cage were laid back. The xiphoid process was grasped with a pair of curved forceps, and the rib cage was raised slightly to facilitate a single incision through it with a pair of large scissors. The heart was removed with the curved forceps, and pressed between two blocks of dry ice. The interval from the time that the chest cavity was opened to the time when the heart was placed on the dry ice was measured with a stopwatch. For intervals from 1.8 to 7.5 seconds, no differences were evident in the concentrations of glycogen within each strain. The removal time of the heart in these studies ranged from 2 to 5 seconds. While still frozen, the heart was scraped clean, and then weighed to the nearest 0.1 mg. on a torsion balance. The other tissues were removed after the heart and in the order: liver, gastrocnemius muscle.

Analytical procedures. The methods for glycogen determination have been described (6). The results are expressed as mg. of glucose per gm. of wet weight of tissue.

Growth hormone. The purified bovine growth hormone used in these studies was prepared in this laboratory by the method of Wilhelmi, Fishman and Russell (12). Slightly alkaline, saline solutions were prepared the day that they were used. All injections were made intraperitoneally (as a single injection) in volumes of 0.05 to 0.25 ml. at a dose of 2 mg. per 100 gm. of body weight.

Insulin. The insulin used in these studies was a commercial preparation, Iletin, Eli Lilly and Company. It was given intraperitoneally in a single dose, 0.1 U. per kg. of body weight, in volumes of 0.15 to 0.25 ml.

Glucose. This was given intraperitoneally in a single dose, 3 mg. per gm. of body weight, in volumes of 0.15 to 0.25 ml.

RESULTS

A. *Studies with young and old mice.* In preliminary studies it became apparent that the levels of cardiac glycogen varied with the age of the animal. A series of observations on male and female mice of the A, C_3H, and I strains established that cardiac glycogen did not increase during a fast if the animals were over 8 months of age (Table 1). In fact, a slight decrease was observed in all cases, but the differences were not significant. The values among males and females, and among the three strains were remarkably similar (Table 1).

In young mice (40–50 days of age), cardiac glycogen did increase during a fast (Table 1). At this age, the early rapid growth phase is nearly completed. The fact that increases in cardiac glycogen did occur in young and not in older mice may be related to the consistently lower levels in the fed state among the young mice; the levels in the fasted state were nearly the same in the two age groups. Greater increments were found in mice of the C_{57} strain (76%) than in mice of the I (38%) or A (31%) strains. Of the three strains, the A exhibits the highest growth rate (2).

TABLE 1. THE EFFECT OF FASTING ON CARDIAC GLYCOGEN IN OLD AND YOUNG MICE

Mice		Non-fasted		Fasted#	
Strain	Sex	Body wt., gm.	Glycogen‡	Body wt., gm.	Glycogen‡
		8–13 month old mice			
A	male	29.8	2.5±0.14 (6)*	25.3	2.3±0.06 (7)
C_3H	male	35.7	2.6±0.24 (5)	30.4	2.3±0.18 (5)
C_3H	female	34.5	2.5±0.15 (7)	31.6	2.3±0.17 (7)
I	male	27.8	2.4±0.30 (6)	24.0	2.0±0.25 (6)
I	female	29.6	2.6±0.15 (8)	27.3	2.3±0.13 (5)
		40–50 day old mice			
A	male	20.3	1.6±0.22 (7)	16.5	2.1±0.10† (7)
C_{57}	male	20.5	1.5±0.12 (8)	15.3	2.7±0.13† (9)
I	male	16.7	1.8±0.17 (6)	12.4	2.5±0.12** (8)

‡ Glycogen, mg./gm. of wet tissue weight and standard error.
* Number of mice.
\# Twenty-four hour fast.
† P value for the differences between the fed and fasted mice, <0.01.
** P value for the difference between the fed and fasted mice, 0.06.

B. *Studies with young-adult mice.* In young-adult mice (3–5 months of age) of the A and C_{57} strains increases in cardiac glycogen also occurred during a fast. The changes in cardiac glycogen during the fasting period for C_{57} strain mice are plotted against time in Figure 1. In the first 12 hours of the fasting period only modest changes were evident, but after this period of quiescence, the concentration of cardiac glycogen increased sharply. The changes in cardiac glycogen of the A strain were similar to those of the C_{57} strain, and for clarity they are not shown in Figure 1. Over the 24-hour fasting period, cardiac glycogen increased in A strain mice from 1.5 ± 0.12 (S.E.) mg./gm., 10 mice, to 2.2 ± 0.14 (S.E.) mg./gm.,

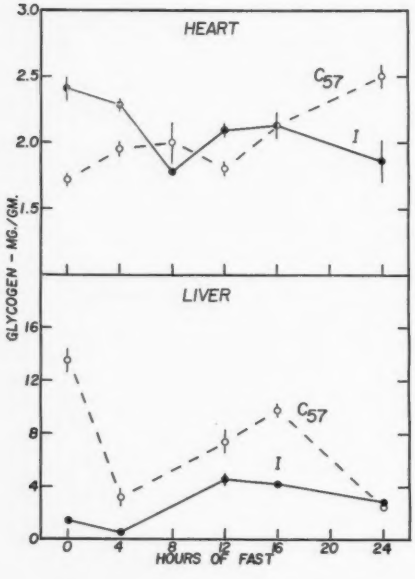

FIG. 1. Effect of fasting on cardiac and liver glycogen of C_{57} and I strain mice. The vertical bars represent the standard errors of the means of 5–16 determinations for the hearts, or 5–10 determinations for the livers. The fast began about 4:30 P.M.

6 mice, and the increase was significant ($P < 0.01$). The increment, about 50%, found in these two strains is comparable to that reported for the rat (9), although the absolute concentrations of glycogen are lower in the mouse.

In contrast to the A and C_{57} strains, a significant ($P < 0.01$) depletion in cardiac glycogen was found in the I strain after 8 hours of fasting, and as the fast continued no further changes occurred (Fig. 1). The concentration of glycogen in the heart of non-fasted I strain mice was greater ($P < 0.01$) than that in A or C_{57} strain mice.

The marked differences in the concentrations of liver glycogen in the I and the C_{57} strains during the experimental period (Fig. 1) suggested that physiological differences, possibly related to liver glycogen, might account for the behavior of cardiac glycogen in the I strain. At the beginning of the

fast, the stores of liver glycogen were greater in the C_{57} strain, and during the latter part of the fasting period, the increases were greater in the C_{57} than in the I strain (Fig. 1). In order to elevate liver glycogen in the I strain, the feeding time was altered from the evening to the daylight hours just prior to the beginning of the fast. Mice of the I and C_{57} strains were fasted for 24 hours, and then fed for 8–9 hours. At the end of this feeding period, about 4:30 P.M., the second 24-hour fasting period was initiated. The levels of cardiac and liver glycogen during this second fasting period are given in Figure 2. The initial concentrations of liver glycogen were 29 times greater in the I strain and 6 times greater in the C_{57} strain than when

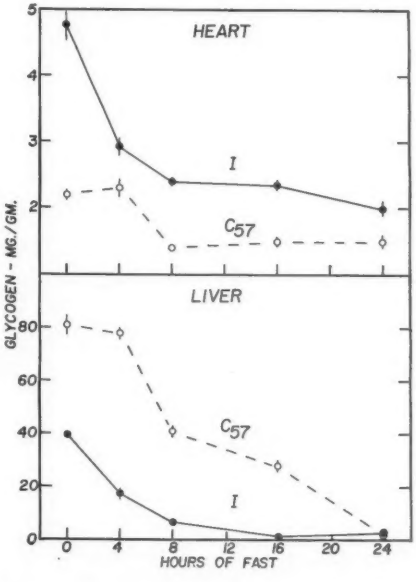

FIG. 2. Effect of fasting on cardiac and liver glycogen of C_{57} and I strain mice after preliminary fasting and feeding periods. These mice were first fasted for 24 hours, then fed for 9 hours before the beginning of the second fasting period at 4:30 P.M. The vertical bars represent the standard errors of the means of 5–8 determinations.

the mice were permitted to eat *ad libitum* (Fig. 1). The initial concentrations of glycogen in the hearts of the two strains were also greater ($P < 0.01$) when the animals were forced to eat during the day (Fig. 2). Nevertheless, significant ($P < 0.01$) decrements in cardiac glycogen were evident in both strains within 8 hours of fasting (Fig. 2). The rate of decline in the I strain was even greater when the initial levels of cardiac glycogen were higher (Figs. 1 and 2).

C. *Studies with homogenates of mouse pituitary gland.* Utilizing the changes in cardiac glycogen as an assay, an attempt was made to detect strain differences in the stores of growth hormone. Pituitary glands were removed from C_{57}, C_3H and I strain mice under nembutal anesthesia. Glands from each strain were homogenized in ice-cold saline; the final pH of the homogenate was 7.9. Homogenates from each strain, containing

about one mouse pituitary gland in a volume of 0.2 ml., were administered intraperitoneally to non-fasted, young-adult C_{57} strain mice at about 9:00 A.M. After a fast of 6 hours, the concentrations of glycogen in the heart, the liver and the gastrocnemius muscle were 1.4 to 2.4 times greater than in untreated mice fasted for the same length of time (Table 2). The pituitary homogenates from each strain evoked similar increases.

D. *Studies with purified growth hormone.* The increases in cardiac glycogen found after the administration of homogenates of mouse pituitary glands (Table 2), were also found after the administration of purified preparations (12) of bovine growth hormone. In this study (Fig. 3), the hormone was given in a single dose (0.2 mg. per 10 gm. of body weight) to non-

TABLE 2. THE EFFECT OF HOMOGENIZED MOUSE PITUITARY GLANDS ON MUSCLE GLYCOGEN LEVELS OF C_{57} STRAIN MICE

Donor mice		C_{57} strain recipients		
		Glycogen, mg./gm.		
Strain	Body wt., gm.	Heart	Gastrocnemius	Liver
C_{57}	25.2	2.9*	4.6*	25.0*
C_3H	30.0	2.6	4.3	38.8
I	22.8	2.8	3.9	49.7
Total Average		2.8 ± 0.42#	4.3 ± 0.30	37.8 ± 5.67
Untreated C_{57} strain mice		1.6 ± 0.11 (9)†	3.1 ± 0.15 (9)	15.8 ± 4.28 (9)
P**		<0.01	<0.01	<0.01

* Each value is the mean of two determinations.
\# Standard error of the mean.
† Number of mice.
** P value for the difference between the injected and the non-injected mice.

fasted mice of the C_{57} strain at about 9:00 A.M., and the mice were fasted for periods up to 6 hours. Within 3 hours the stores of glycogen in the heart were doubled, whereas only minimal changes occurred in the gastrocnemius muscle or the liver (Fig. 3). It is interesting that this dose of purified bovine growth hormone (0.4–0.5 mg.) was no more effective on the stores of cardiac glycogen than a single mouse pituitary gland, wet weight 1–2 mg. (Table 2 and Fig. 3).

In another series of experiments, growth hormone was given to non-fasted mice of the C_{57} strain at about 4:30 P.M. After a fasting period of 4 hours, the stores of cardiac glycogen were no greater than those in untreated mice (Table 3, Part A). This failure in the activity of the hormone was puzzling, since dramatic effects were seen if the hormone was given only 7 hours earlier in the day (Fig. 3).

One possible explanation for this failure was the obvious difference in the physiological states of the mice at the two times of the day. Mice eat very little during the day, and the stores of liver glycogen deplete rapidly from 9:00 A.M. to 4:30 P.M. (Figs. 1 and 3). In order to mimic the physiological state at 9:00 A.M., mice were given intraperitoneal injections of

glucose (Table 3, Part B), or were forced to eat during the day (Table 3, Part C), before growth hormone was administered at 4:30 P.M. The maximum effect of either treatment was a slight increase (20%) in activity of the hormone (Table 3, B-3 and C-2). However, if the same dose of glucose was superimposed upon the forced feeding regime, then the effect of the hormone seen after dosage at 9:00 A.M. could be duplicated (Table 3, C-3). This same response could be elicited if insulin was given prior to or

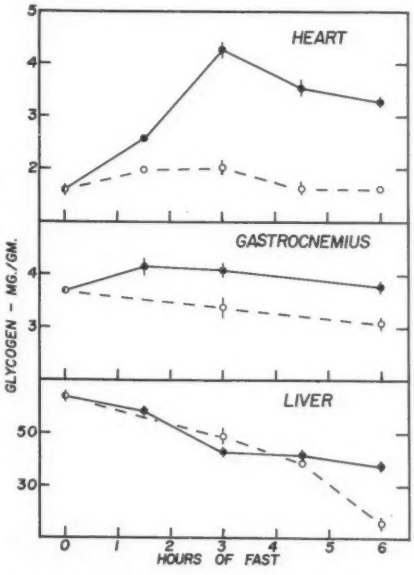

FIG. 3. Effect of growth hormone on muscle and liver glycogen of C_{57} strain mice. The solid lines represent mice injected with growth hormone (0.2 mg. per 10 gm. of body weight) at 9:00 A.M. and then fasted. The dashed lines represent fasted, untreated mice. The vertical bars represent the standard errors of the means of 5–10 determinations per point.

simultaneously with growth hormone at 4:30 P.M. (Table 3, D-2). Insulin given after growth hormone was less effective (Table 3, D-3), and insulin alone had no effect (Table 3, D-1).

In the young-adult I strain mouse, cardiac glycogen decreased during a fast (Figs. 1 and 2). Attempts were made to reverse this depletion by administering purified growth hormone. Ten young-adult I strain mice were given a dose of growth hormone (0.2 mg. per 10 gm. of body weight) at 4:30 P.M., and fasted for 4 hours. The average concentration of cardiac glycogen was 2.4 ± 0.18 (S.E.) mg./gm., which was no different from that found in untreated mice (Fig. 1), or mice receiving injections of saline. A second group of 10 I strain mice were fasted for 24 hours, then fed for 8–9 hours before receiving the same dose of growth hormone at 4:30 P.M. After a 4 hour fast, the average concentration of cardiac glycogen was 3.9 ± 0.23 (S.E.) mg./gm. This value is about midway between the initial level and that found after a 4 hour fast of untreated mice forced to eat during the day (Fig. 2).

TABLE 3. THE EFFECT OF GROWTH HORMONE ON CARDIAC GLYCOGEN IN C_{57} STRAIN MICE. GROWTH HORMONE WAS GIVEN AT 4:30 P.M. TO THOSE GROUPS MARKED WITH AN (*), AND IN ALL CASES THE MICE WERE FASTED FROM 4:30 TO 8:30 P.M.

Experimental conditions	No. of mice	Glycogen		P_1	P_2
		Heart	Liver		
		mg./gm. of tissue			
A. The effect of growth hormone (G.H.)					
1. No treatment	9	2.0±0.12#	3.2±1.4	—	—
(*) 2. Growth hormone	12	2.4±0.15	5.6±1.9	0.07	—
B. The effect of a dose of glucose given prior to G.H.					
1. Glucose only, 4 hrs. before fasting	5	2.2±0.15	—	0.5	—
(*) 2. Glucose, 1–3 hrs. before G.H.	6	2.5±0.24	7.0±2.2	0.04	—
(*) 3. Glucose, 4 hrs. before G.H.	6	2.9±0.14	5.2±1.2	<0.01	0.04
C. The effect of an increased alimentation† prior to G.H.					
1. No treatment	6	2.3±0.26	77.6±4.7	0.19	—
(*) 2. G.H.	6	2.8±0.15	80.4±6.3	<0.01	0.07
(*) 3. Glucose, 4 hrs. before G.H.	6	3.5±0.44	71.9±3.9	<0.01	<0.01
4. Glucose, 4 hrs. before fasting	6	2.3±0.26	69.7±6.4	0.14	—
D. The effect of insulin given before, with or after G.H.					
1. Insulin only	5	1.7±0.17	—	—	—
(*) 2. Insulin, 1 hr. before or simultaneously with G.H.	6	3.9±0.17	—	<0.01	<0.01
(*) 3. Insulin, 1 hr. after G.H.	3	3.1±0.09	—	<0.01	0.05

Standard error of the mean.
P_1, P value for differences in cardiac glycogen from A-1.
P_2, P value for differences in cardiac glycogen from A-2.
† All mice in this group were fasted for 24 hours, then fed for 8–9 hours before the beginning of the second fast at 4:30 P.M.

We have recently reported (13) that the I strain mouse exhibits decreasing stores of vitamin B_6 with age. Therefore, aseries of I and C_{57} strain mice were fed the stock ration supplemented with 50 mg. of pyridoxine hydrochloride per kg. of diet for 2 weeks. The concentrations of cardiac glycogen in the two strains during a 24-hour fasting period were no different from those shown in Figure 1.

DISCUSSION

If differences in the peripheral activity of growth hormone do exist in these strains of mice, they are certainly not discernible from the changes in cardiac glycogen. For example, the increments in cardiac glycogen during a fast were greater in young C_{57} than in young A strain mice (Table 1), but in young-adult mice the increments were similar in the two strains (see section B). If the increments in cardiac glycogen during a fast are solely the reflection of the titers of growth hormone, then it is difficult to explain the well-established observation that A strain mice grow more rapidly than C_{57} strain mice (2). The behavior of cardiac glycogen during a fast in the I strain presents an even more extreme picture; sizeable in-

creases were observed in young mice (Table 1), but decreases were observed in young-adult mice (Figs. 1 and 2), and no changes were observed in older mice, as found with the other strains (Table 1).

The decrease in cardiac glycogen in the fasting young-adult I strain mouse does not seem to be due to a deficiency in the pituitary stores of growth hormone (Table 2), nor did the administration of rather large doses of bovine growth hormone appreciably alter the depletion of cardiac glycogen during a fast (see section D). In view of the fact that the I strain mouse has an unusual requirement for pyridoxine (13), it was surprising that large supplements of pyridoxine (50 mg. per kg. of diet), did not alter the behavior of cardiac glycogen (Fig. 1). However, experiments now in progress suggest that the time of feeding of the pyridoxine supplements was not optimal, and this point is still under investigation. Since Cori and Illingworth (14) have demonstrated that pyridoxal phosphate is the coenzyme of phosphorylase, and since it has been found that I strain mice fed a complete ration exhibit lower levels of vitamin B_6 at 100 than at 60 days of age (13), an explanation of the unique decrease in cardiac glycogen during a fast in terms of the vitamin B_6 metabolism offers some promise. It is evident that the large stores of muscle glycogen found in the I strain (3, 4), and their maintenance during a fast, cannot be due to an excessive output of growth hormone.

The data presented in Figures 1 and 2 and in Tables 1 and 2 demonstrate that the concentrations and the changes in cardiac glycogen in the mouse must be related to the age of the animal. Secondly, the stores of cardiac glycogen must be subject to a variety of controls which in turn vary with the age and the strain of the mouse.

One of these controls would appear to be a requirement for the presence of insulin for the action of growth hormone on cardiac glycogen. The administration of growth hormone to well-fed mice (at 9:00 A.M.) yielded increases of two-fold in the stores of cardiac glycogen (Fig. 3), but a total of four trials, using several experimental conditions, failed to yield comparable increases if the mice were injected at 4:30 P.M. (Table 3). The increment found in the mice injected in the morning could be duplicated if the animals were pretreated with insulin (0.1 U./kg.), or with a dose of glucose and forced alimentation (Table 3, C-3 and D-2). It is tempting to speculate that the peripheral activity of insulin is high at 9:00 A.M., following the large food intake during the night, and that the peripheral supply of insulin is low at 4:30 P.M. because mice feed only sporadically during the day, or that a reduced output of insulin during the day is the result of rhythmical controls. It is of interest here also that cardiac glycogen did not increase in the fasting mouse until the stores of liver glycogen began to increase (Fig. 1).

The fact that a dose of glucose superimposed upon the forced alimentation (Table 3, C-3) was more effective than forced alimentation alone may

be explained in several ways. It is possible, for example, that the single dose of glucose supplied a greater stimulus to the pancreas than a slow absorption of glucose from the intestine. This requirement of insulin for the action of growth hormone receives some support from the work of Russell and Bloom (9), who found larger increments in cardiac glycogen if glucose was given in addition to growth hormone to a fasting rat. It might be argued that since growth hormone is as effective in an alloxan diabetic rat as in a normal rat, that insulin is not required (8). However, the alloxan diabetic rat must have some stores of insulin because a nearly total pancreatectomy of a rat will produce death within 48 hours unless insulin is supplied (15).

Acknowledgment

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REFERENCES

1. Fenton, P. F. and M. T. Dowling: *J. Nutrition* **49**: 319. 1953.
2. Fenton, P. F. and C. J. Carr: *J. Nutrition* **43**: 441. 1951.
3. Lyon, J. B., Jr.: *Am. J. Physiol.* **190**: 434. 1957.
4. Lyon, J. B., Jr. and P. F. Fenton: *Amer. J. Physiol.* **187**: 415. 1956.
5. Lyon, J. B., Jr.: *Fed. Proc.* **16**: 215. 1957.
6. Lyon, J. B., Jr.: *Am. J. Physiol.* (In press).
7. Fenton, P. F. and J. M. Marsh: *J. Nutrition* **60**: 465. 1956.
8. Illingworth, B. A. and J. A. Russell: *Endocrinology* **48**: 423. 1951.
9. Russell, J. A. and W. L. Bloom: *Endocrinology* **58**: 83. 1956.
10. Adrouny, G. A. and J. A. Russell: *Endocrinology* **59**: 241. 1956.
11. Bloom, W. L.: *Am. J. Physiol.* **186**: 518. 1956.
12. Wilhelmi, A. E., J. B. Fishman and J. A. Russell: *J. Biol. Chem.* **176**: 735. 1948
13. Lyon, J. B., Jr., H. L. Williams and E. A. Arnold: *J. Nutrition* 66:261. 1958.
14. Cori, C. F. and B. A. Illingworth: *Proc. Nat. Acad. Sci.* **43**: 547. 1957.
15. Scow, R. O.: *Endocrinology* **60**: 359. 1957.

THE LOCALIZATION OF I^{131}-LABELED TRIIODOTHYRONINE IN THE PITUITARY AND BRAIN OF NORMAL AND THYROIDECTOMIZED MALE RATS

DONALD H. FORD, STRATOS KANTOUNIS AND ROBERT LAWRENCE

Department of Anatomy, State University of New York, Downstate Medical Center, Brooklyn, New York

ABSTRACT

The central nervous system and pituitary lobes of normal and hypothyroid male rats were both shown to be capable of concentrating triiodothyronine following intravenous injection of I^{131}-labeled triiodothyronine. The pituitary site of maximal localization was similar to that observed in the guinea pig, being greatest in the anterior lobe.

An autoradiographic study of the central nervous system and pituitary lobes showed a localization pattern similar to that seen in the guinea pig, being primarily associated with nuclear or cellular areas. However, the pronounced localization seen in the regions occupied by the neurosecretory structures in the guinea pig and rabbit was not observed in the rat. In addition, it was noted that the hippocampus, an area not actually studied in the other species, was practically autoradiographically negative in the rat.

Thyroidectomy decreased the rate of uptake of labeled hormone in the central nervous system. However, the peak concentration was increased in the central nervous system as well as in the adenohypophysis. The delay in attaining a peak concentration and the increased amount taken up were attributed to a deficiency in deiodinating mechanisms. A possible role for the thyroid hormone in maintaining cellular enzyme systems is suggested.

A possible relationship between the uptake curves of labelled triiodothyronine and the conjugated form of triiodothyronine is discussed.

Injections with radio iodinated serum albumen showed that the vascular space of the rat brain is very small, similar to the rabbit, and somewhat enlarged by hypothyroidism.

THE selective accumulation of I^{131}-labeled thyroxine and triiodothyronine by the pituitary and central nervous system (CNS) of the rabbit and guinea pig has been shown by several investigators (1, 2, and 3). In both species, the uptake of triiodothyronine was greater than the uptake of thyroxine under similar conditions, and it was observed that the pituitary lobe which concentrated the most triiodothyronine differed between species, the anterior lobe being most active in the guinea pig and least active in the rabbit. In investigations (3) on rats injected with labeled

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thyroxine, no observable difference between the two lobes in their affinity for the labeled hormone was observed, and they concentrated even less than did the median eminence. It was also observed that thyroidectomy had little or no effect on the organ/plasma ratios. Inasmuch as these experiments were done with thyroxine which is relatively poorly concentrated by both the brain and pituitary and since only one time interval after injection was used, it is difficult to assess what, if any, effect the thyroidectomy actually had, particularly since it would appear that the normal animals failed to concentrate thyroxine.

In view of these negative experiments with thyroxine in the rat and in consideration of the greater affinity of neural and pituitary tissues for triiodothyronine, the following experiments were performed with the objective of ascertaining, first of all: if the rat, like the rabbit and guinea pig, can localize and concentrate triiodothyronine within the pituitary and CNS; 2) whether the pituitary localizes the labeled hormone in a manner similar to the rabbit or to the guinea pig; and 3) what would be the effect of hypothyroidism on the uptake of thyroid hormone by the neural and pituitary tissues.

MATERIALS AND METHODS

The I^{131}-labeled triiodothyronine used in these experiments was obtained from the Oak Ridge laboratory of the Abbott Corporation, in a 50% propylene glycol solution. The material was checked for purity chromatographically and found to have a mean purity of 80% (72–87%). The method of dilution and chromatographic analysis has been previously described (1).

Male rats of the Wistar strain weighing from 295 gm. to 380 gm. were used in this investigation. There were 33 normal and 31 surgically thyroidectomized animals. The completeness of the thyroidectomy was checked by counting the amount of free iodide (131) taken up in the region of the thyroidal trachea after the animals injected with the labelled triiodothyronine were killed. If there was an appreciable uptake, indicating the presence of thyroidal tissue, the animal was discarded.

A third group consisted of 20 sham-operated animals. Inasmuch as the data obtained from this group was the same as that from normal animals, it will not be included.

The normal, thyroidectomized and sham-operated animals all received a dose of 0.5 μg. of triiodothyronine/kilogram (approximately 34 μc./kg.), intravenously into the jugular vein.

In addition to the above groups, a few animals were injected with a dose of 1 mc./kg. (10.5–27.5 μg./kg.). Four animals were used for an autoradiographic study, while 5 normal and 2 thyroidectomized animals provided fresh tissues for chromatographic analysis. Finally, 3 normal and 3 thyroidectomized animals were injected with 42 μc./kg. of Radio-Iodinated Serum Albumen (RISA) to obtain information relative to the vascular space in the various parts of the CNS studied.

All animals were maintained on a standard laboratory chow and water, *ad libitum*. The thyroidectomized animals were also given sufficient calcium lactate in their water to provide for a 1% solution. The thyroidectomy was performed from four to six weeks prior to the time of injection. The time intervals at which the animals were killed after injection were ½, 1, 2, 4 and 7 hours. The injection, counting, chromatographic and autoradiographic procedures were the same as those previously employed (1, 4).

Since one purpose of the investigation was to observe the effect of hypothyroidism on the ability of the pituitary and CNS to concentrate the labeled triiodothyronine, a statistical evaluation of the data was made by the method of Student "t" (5). However, it should be pointed out that there is an error of about 5% which occurs when counting low levels of radioactivity with the scintillation counters used to obtain the data. The addition of this error to the normal biological variation may mask or accentuate statistical differences. In view of this consideration, perhaps more attention should be directed to the shape and direction of the curves obtained than to the actual statistical differences between isolated points on the curves.

RESULTS

In the normal rat, the rate of clearance of I^{131} from the plasma after intravenous injection with I^{131}-labeled triiodothyronine was very rapid during the first $\frac{1}{2}$ hour and much slower during the next six and one-half hours (Fig. 1). Thyroidectomized animals showed a similar rapid clearing of I^{131} from the plasma during the first $\frac{1}{2}$ hour, but the level of radioactivity in the blood after this period remained at a slightly higher level than in the

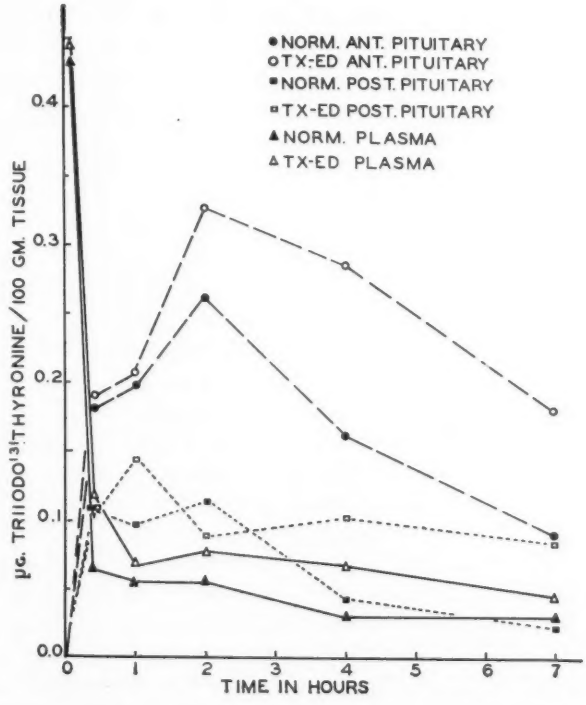

FIG. 1. The concentration of I^{131} in the pituitary and plasma at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals while the hollow symbols represent the values obtained from thyroidectomized animals.

normals. This difference was approximately 0.035 μg. of triiodothyronine /100 gm. of plasma for the last three hours. This difference was significant 4 hours after the time of injection ($P < 0.01$).

As the concentration of radioactivity in the plasma fell, there was a rapid uptake in both pituitary lobes, which was highest for the anterior lobe. The greatest concentration was attained 2 hours after injection, following which there was a slow decrease in the radioactivity present in both lobes. After thyroidectomy, the rate of uptake in the anterior pituitary was about the same during the first hour, with a slight increase in rate of uptake during the second hour. At the time of greatest triiodothyronine concentration there was 0.063 μg./100 gm. of tissue more in the thyroidectomized animals. The subsequent rate of fall in concentration closely paralleled that of the normal animals, but was maintained at a higher level. This difference was significant 4 hours after the injection of the hormone ($P < 0.01$). Note that in both the normal and thyroidectomized groups, there was a slight drop in the rate of uptake between the $\frac{1}{2}$- and 1-hour period.

The posterior pituitary lobes in both the normal and thyroidectomized groups reached their maximal concentration at lower levels than did the anterior lobes, the time of greatest concentration being between the first and second hour. At no time during the course of the experiment was there an appreciable difference between the two groups.

In the CNS of normal animals, the rate of uptake of I^{131} after the injection of I^{131}-labelled triiodothyronine was relatively fast during the first $\frac{1}{2}$ hour, during a period in which the plasma values were falling (Figs. 2 and 3). This was followed by a definite lag in the rate of uptake for the second $\frac{1}{2}$ hour, after which there was again a rapid increase in the concentration of radioactivity within the tissues, with maximal concentration occurring at the end of 2 hours. The CNS concentration then fell slowly for the following 2 hours after which the concentration remained relatively constant, paralleling the concentration within the plasma.

In the thyroidectomized animals, the rate of uptake in the CNS during the initial 2 hours was for all intents and purposes identical to that of the normal animals (Figs. 2 and 3), with the exception of the isolated mammillary body in which no lag in rate of uptake was observed during the second $\frac{1}{2}$-hour period. However, the highest concentration was not reached until 4 hours after injection in these animals. This concentration in the most responsive tissue (mammillary body) averaged 0.009 μg. of triiodothyronine/100 gm. tissue higher than that observed in the normal mammillary body (range 0.004–0.014), and the normal neuronal cellular area from which it differed most was the cerebral gray. The difference in the concentration of radioactivity within isolated mammillary body of the normal and thyroidectomized animals was significant one hour after the injection ($P < 0.05$). During the last 3 hours of the experiment, the con-

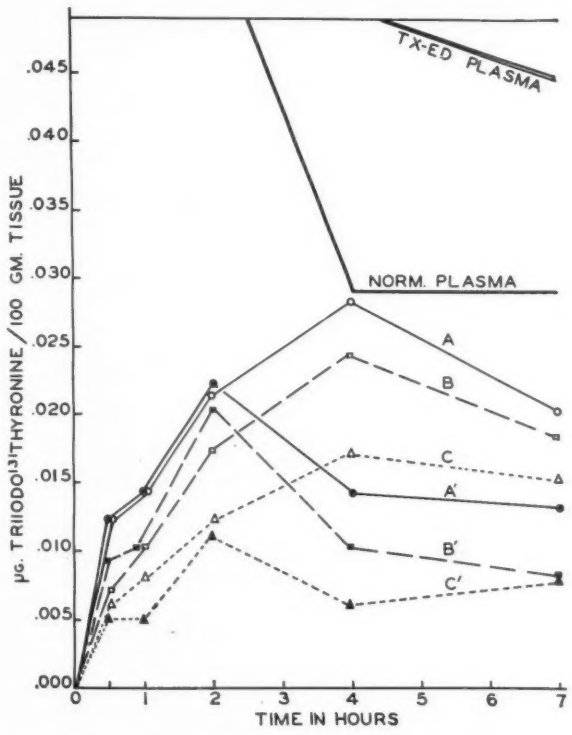

FIG. 2. The concentration of I^{131} in the cerebellum, cerebral gray and cerebral white matter at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals while the hollow symbols represent values from thyroidectomized animals. The circles indicate the cerebellum (A,A^1), the squares the cerebral gray (B,B^1), and the triangles the cerebral white matter (C,C^1).

centration of radioactivity in the neural tissues of thyroidectomized animals fell at a rate comparable to the rate of fall in the plasma level of radioactivity. This rate appeared to be somewhat faster than that observed in the normal animals, where the rate of fall, while less, also paralleled the concentration maintained in the plasma.

It is to be noted in both normal and thyroidectomized animals, that the concentration of radioactivity was greater in the cerebral gray matter than in the white matter, being significantly higher ($P < 0.05$) in the normal group 1 and 4 hours after the injection.

Chromatographic separation of normal plasma samples showed that as the concentration of radioactivity in the plasma drops sharply with time (Fig. 1), the level of radioactivity attributable to triiodothyronine also dropped, with the greatest rate of fall occurring in the first one hour (Fig.

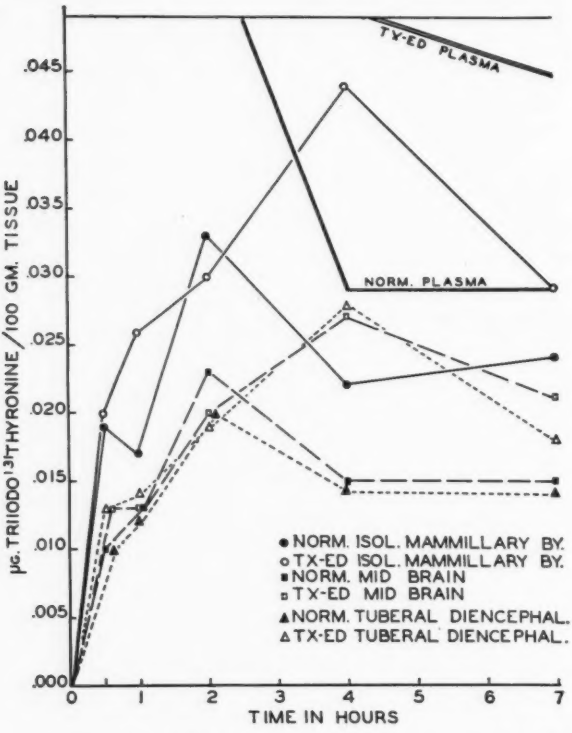

FIG. 3. The concentration of I^{131} in the isolated (isol.) mammillary body (by.), mid brain and tuberal diencephalon (diencephal.) at various time intervals after the intravenous injection of 0.5 μg./kg. of triiodothyronine. The solid symbols represent values from normal animals, while the hollow symbols represent values from thyroidectomized animals.

4). This drop in plasma triiodothyronine was accompanied by an increasing per cent of the total plasma radioactivity which could be attributed to free iodide. A somewhat different picture was presented by the thyroidectomized group, where the per cent of total plasma radioactivity attributable to triiodothyronine fell off much less and was accompanied by a much smaller increase in plasma iodide levels.

The more rapid rate of deiodination of triiodothyronine observed in the plasma of normal animals was also seen in the adenohypophysis and in the CNS (Table 1). These differences in the percents of the various forms in which the radioactivity was present are considered to indicate some difference in the abilities of the adenohypophysis and CNS of the two groups to handle the injected material. With the exception of the posterior pituitary, the tissues in the normal group had less triiodothyronine and more iodide than did comparable tissues in the thyroidectomized group. There

TABLE 1. THE METABOLITES OF TRIIODO^{131} THYRONINE PRESENT IN THE PITUITARY, BRAIN AND PLASMA OF THE NORMAL AND HYPOTHYROID MALE RAT AFTER INTRAVENOUS INJECTION OF 1.0 MC./KG.* OF THE LABELED HORMONE

Tissue	Triiodothyronine		Iodide		Residue	
	N	Tx	N	Tx	N	Tx
1 Min Plas	78.0†	76.0†	4.0	4.1	7.3	17.2
2 Hour Plas	42.7	57.1	26.0	15.5	28.9	25.0
Ant. P.t.	57.5	65.7	7.6	6.0	29.4	25.3
Post P.t.	55.4	39.7	2.9	11.0	33.0	37.2
Iso. Mam. Body	55.0	73.2	10.7	5.4	26.8	18.6
Iso. Tub. Cin.	34.1	58.3	23.9	10.4	35.3	24.8
Cerebral Grey	30.6	69.0	11.8	4.2	41.7	23.4
Cerebral White	38.2	59.9	12.1	9.8	35.2	27.2
Cerebellum	61.1	65.5	4.6	6.4	29.0	24.3

N = Normal, Tx = Thyroidectomized.

* Dose of Triiodothyronine = 25.0 μg./kg. for normal rat and 21.6 μg./kg. for the thyroidectomized rat.

† Sum of 6 animals receiving 0.5 μg./kg.

was also a greater I^{131} per cent listed as Residue (unidentified iodine-containing substances) found in the tissues of normal animals.

Since the plasma of the thyroidectomized group maintained a higher level of total radioactivity than that of the normals, the increased radio-

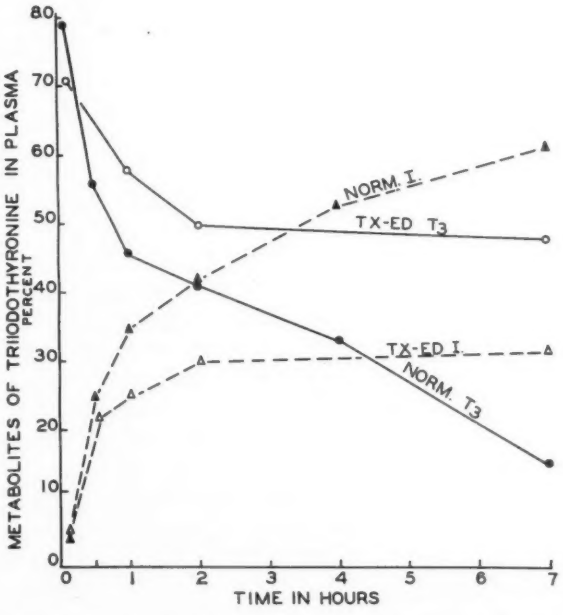

FIG. 4. A graphic representation of a chromatographic analysis of the distribution of the metabolites of I^{131}-labeled triiodothyronine in the plasma of normal and thyroidectomized animals after the intravenous injection of 1 mc. of the labeled hormone/kg. (Norm. = normal, Tx-ed = thyroidectomized, I = iodide, and T_3 = triiodothyronine).

activity in the tissues of the thyroidectomized animals might be due to the increased radioactivity in the trapped blood. If true, the time of highest concentration in the CNS and pituitary of the thyroidectomized animals would be expected to occur at the same time as in the normals. Even though the times at which the maximal concentration occurred differed, it was possible that a considerable portion of the increased activity in tissues from thyroidecomized animals might be due to the radioactivity of the trapped blood.

The experiment with RISA injected animals (Table 2) showed that the radioactivity in the trapped tissue plasma was not great enough to account for the increased radioactivity in the thyroidectomized group, despite an increase in the CNS blood volume which occurred in most of the CNS in

TABLE 2. A COMPARISON OF THE CONCENTRATION OF I^{131}-LABELED TRIIODOTHYRONINE (0.5 μG./KG.) (−34.0 μC./KG.) AND RADIO IODINATED SERUM ALBUMEN (RISA) (42 μC./KG.) IN THE CNS AND PITUITARY OF NORMAL AND THYROIDECTOMIZED RATS AS REFLECTED BY THE ORGAN/PLASMA RATIO

	Triiodothyronine One hour after injection		RISA One and one-quarter hours after injection (=plasma space)		
	Normal	Thyroid-ectomized	Normal	Thyroid-ectomized	Change after Thyroidec-tomy
Ant. Pituitary	3.45	2.87	0.089	0.086	−0.003
Post. Pituitary	1.89	1.88	0.093	0.048	−0.045
Isol. Mammillary Body	0.311	0.278	0.0057	0.0170	+0.0113
Isol. Tuber Cinereum	0.284	0.264	0.0077	0.0110	+0.0033
Tuberal Diencephalon	0.179	0.192	0.0052	0.0050	−0.0002
Chiasmic Diencephalon	0.181	0.171	0.0056	0.0063	+0.0007
Cerebellum	0.249	0.202	0.0078	0.0150	+0.0072
Cerebrum	0.150	0.146	0.0054	0.0090	+0.0036
Cerebral Gray	0.168	0.157	0.0075	0.0126	+0.0051
Cerebral White	0.088	0.113	0.0049	0.0063	+0.0014

the thyroidectomized animals (Table 2): (i.e., calculation of the increase in the vascular space of the isolated mammillary body, over that normally found, showed it to be 0.0113%. When this is multiplied by the difference in blood level of radioactivity at the time of greatest divergence (0.035 μg. at 4 hours), one observes that the 0.00040 μg. of triiodothyronine/100 gm. of mammillary body which would have to be added to the normal or subtracted from the thyroidectomized animals to correct for differences in blood volume or total plasma radioactivity, would in no way change the result).

While the plasma volume changes in the CNS of thyroidectomized animals were in all but one instance positive, the changes in the pituitary were negative, and it is interesting to note that the one part of the CNS which showed a slight decrease in plasma volume is associated with the posterior pituitary.

The autoradiographic localization of what was shown to be at least part-

ly triiodothyronine (Table 1) and its metabolic breakdown products was within those parts of the CNS most rich in cell bodies, being much more concentrated in the gray than in the white matter. While the pattern of localization was similar to that seen in rabbits and guinea pigs, it was somewhat more diffusely distributed. Other relatively clear nuclear localizations were noted throughout the neuraxis (Fig. 5), with a particularly marked localization in the arcuate nucleus in the median eminence. It is interesting to note that the hippocampus, which possesses a very dense layer of pyramidal cells, was much less reactive than the cerebral cortex. No pronounced accumulations of radioactivity could be associated with the neurosecretory areas, as were shown in the rabbit and guinea pig (1, 2).

DISCUSSION

This investigation was designed to consider three questions, which were: a) will the CNS and pituitary tissues of the rat concentrate triiodothyronine? b) which pituitary lobe will be the most active in concentrating the labeled hormone? c) what will be the effect of hypothyroidism on the ability of these tissues to concentrate the labelled hormone?

There is little doubt but that the CNS and pituitary tissues in the rat, like those of the rabbit and guinea pig, are capable of concentrating L-3:5:3′-triiodothyronine. Furthermore, the labeled hormone was cleared from the plasma at a rate equal to that seen in the rabbit or guinea pig (1, 2). While the organ/plasma ratios never exceeded one in either normal or thyroidectomized animals (Table 2), they still exceeded the plasma and hence the water space of the brain (Table 2); so it must be concluded that the material had at least passed through the capillary walls. Also, since electronmicroscopy of the rat brain (6, 7) reveals practically no extracellular space, it is probably safe to conclude that the radioactive material is in either the neuronal or glial elements. The nature of the material localized in the CNS, as in the guinea pig and rabbit, appeared to be partly triiodothyronine and partly its metabolic breakdown products (Table 1). Inasmuch as free iodide is not taken up by the brain within the time limits of these experiments (2) and apparently must be incorporated into the thyroid hormone before being taken up by neural tissues (8), the iodide observed in the CNS must be derived through intracellular deiodination processes.

The autoradiographic localizations are similar to those observed in the guinea pig and rabbit, although a specific localization in the neurosecretory area was not observed. This may, in part, be related to the fact that there is very little histologically visible neurosecretory product in the rat (9) as compared with either the rabbit (10) or guinea pig (unpublished observation).

The observation that the hippocampus with its densely packed layer of pyramidal cells was autoradiographically negative was surprising. This was

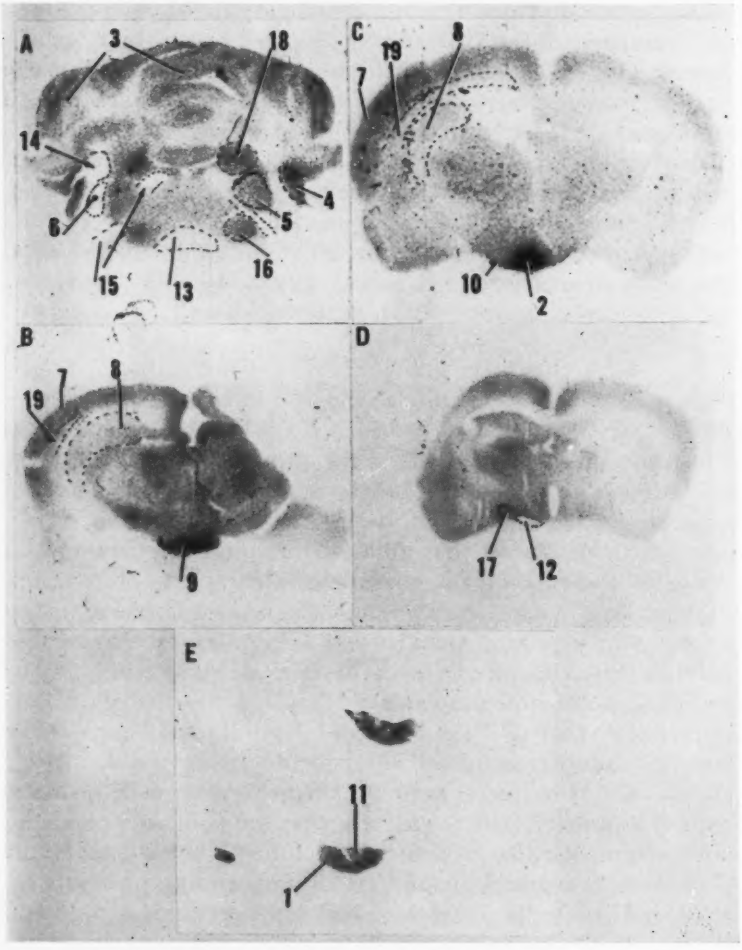

FIG. 5. An autoradiographic representation of the sites of localization of I^{131} in the rat brain after injection of I^{131}-labelled triiodothyronine. A. Coronal section through the cerebellum and pons; B. Coronal section through the caudal diencephalon and associated cerebral hemispheres; C. Coronal section through the median eminence and associated cerebral hemispheres; D. Coronal section through the rostral diencephalon and associated cerebral hemispheres; E. Coronal section through the pituitary. The numbers refer to the following: 1. Adenohypophysis, 2. Arcuate nucleus, 3. Cerebellum, 4. Cochlear nuclei, 5. Descending trigeminal nucleus, 6. Descending trigeminal tract, 7. Gray matter, 8. Hippocampus, 9. Mammillary body, 10. Median eminence, 11. Neurohypophysis, 12. Optic tract, 13. Pons (fibers and nuclei), 14. Restiform body, 15. Seventh nerve (fibers), 16. Seventh nerve (nucleus), 17. Supraoptic nucleus, 18. Vestibular nuclei, 19. White matter.

particularly true since S^{35}-labelled methionine (11) and S^{35}-labeled L-cystine (12) have shown a rather pronounced localization in this band of cells. The observations with the labeled cystine are of further interest in that the autoradiographs were made at various time intervals over a 24-hour period. They showed that the hippocampal pyramidal cell area concentrated the amino acid more rapidly than did the cerebral cortex. It was felt that this indicated differences in the rates of amino acid incorporation into the neural protein in these areas. The observation that triiodothyronine is not well localized in the hippocampus by autoradiographic procedures as compared with the cerebral cortex, also implies some difference

TABLE 3. COMPARISON OF CENTRAL NERVOUS SYSTEM SITES OF DEIODINATING ACTIVITY WITH THE CONCENTRATIONS OF I^{131}-LABELED TRIIODOTHYRONINE IN THE SAME AREAS

Tissue	Deiodinating* activity	μg. Triiodothyronine/100 gm. CNS tissue at different time intervals (in hours)					
		½	1	2	4	7	
Whole Brain	100	—	—	—	—	—†	Tissues high in Deiodinose Activity
Cerebrum	172	.008	.009	.013	.009	.008	
Cerebral Cortex	240	.010	.010	.020	.010	.008	
Cerebellum	82	.012	.014	.022	.014	.013	Tissues low in Deiodinase Activity, but relatively high in concentration of Triiodothyronine
Medulla	37	.012	.013	.023	.013	.014	
Mid Brain	13	.012	.013	.023	.015	.015	
Isolated Mamillary‡ Body	—	.021	.017	.033	.022	.024	

* From Tata (13).
† Not determined in this study.
‡ Not determined in TATA's study.

between the two areas in the rate at which the normal metabolic processes may be proceeding.

In previous papers (1, 2) an attempt was made to correlate the higher localizations of thyroid hormone in the hypothalamus with some specific role which it might play in this area. While this still may be true, there also exists the possibility that the amount localized may depend on the amount of deiodinase present, being low in areas rich in deiodinase. The possibility of such a relationship was suggested by comparing the data on triiodothyronine concentration with Tata's data on deiodinating activity in the brain (13, Table 3). It was noted that areas relatively rich in deiodinating activity, like the cerebral cortex, had a lower concentration of triiodothyronine than did the areas low in deiodinating activity, like the mid brain, where the triiodothyronine concentrations were higher. One would suspect on this basis that the isolated mammillary body might have an even lower concentration of a deiodinating enzyme if this analogy is true. Unfortunately, no information is available for this area. If this relationship is true, the hippocampus might be expected to contain a very high level of deiodinase activity, inasmuch as the autoradiographic concentra-

tion of triiodothyronine appears to be so low in this structure. If the relative concentrations of triiodothyronine in the brain and pituitary do depend primarily on the relative concentrations of deiodinase, one would have to presume that the adenohypohysis, the arcuate nuclei and the neurosecretory region in the rabbit and guinea pig would be practically free of deiodinase. Inasmuch as chromatographic studies have shown that deiodination is occurring in these areas, one must conclude that while the relative concentrations of triiodothyronine which were found may depend, in part, upon the deiodinating activity within that region, this can not be the only factor involved.

The second question pertained to the pituitary site of maximal localization, and it was observed that the anterior lobe, as in the guinea pig, concentrated considerably more of the labeled hormone than did the posterior lobe. Why the rat and the guinea pig differ from the rabbit in this respect is still unknown.

That the thyroid hormone plays some role in the normal function of the brain has long been accepted. However, the nature of this role has been most elusive. There have been many investigations on the utilization of oxygen and glucose (14–17), studies on growth and maturation and vascular supply (18–22), and effects on the EEG pattern and nerve excitability (23–25), yet, the precise manner whereby the thyroid hormone affects normal brain function has not yet been clearly ascertained. Timiras and Woodbury (26, 27), have reported a most interesting series of results which appear rather clearly to implicate the thyroid hormone in aiding the adrenocorticoids in the control of the electrolyte levels within the nerve cells. Thus, the third question dealing with the effects of thyroidectomy relates to the overall question of the role of the thyroid hormone in CNS function.

The two most discernible effects of thyroidectomy were that there was a slight increase in the vascular space in the thyroidectomized animals (Table 2) and that while the tissues of the thyroidectomized animals concentrated more of the labeled hormone (Figs. 1, 2, 3, 4), it has taken them longer to do so. The adenohypophysis is the only real exception in that it achieves its maximal concentration at the same time in both groups. The change in vascular volume induced by hypothyroidism is not surprising since Eayrs (21) has previously shown that hypothyroidism affects the vascular system.

A third observation, based on chromatographic analysis of the tissues and plasma reveals a decrease in deiodination in the thyroidectomized animals (Fig. 4, Table 1). This evidence for a disruption in an enzyme function suggests that one role for the thyroid hormone may be in the maintenance of intracellular enzyme systems. While this is probably not a role restricted to neural tissues, or to deiodinating enzymes alone, nothing further can be said at this time.

While the increase in the concentration of radioactivity in the tissues of thyroidectomized animals may, in part, be due to the higher concentration in the plasma and the slightly increased vascular space in CNS tissues (Table 2), the total increase attributable to these factors was not sufficient to account for the change in concentrations. Also, if these were the only factors involved, one would have suspected that the time of maximal concentration would have coincided with that of the normal animals, as it does in the pituitary. Inasmuch as the space alterations in the pituitary were negative (Table 2) and since the increase in plasma radioactivity was not enough to account for a rise in anterior pituitary radioactivity, some other factor must also be concerned with the increase observed in the adenohypophysis.

Another explanation for an increased concentration of radioactivity in the CNS (3) might be that there is no endogenous thyroid hormone to dilute the intracellular concentration of the labeled hormone, thus increasing the potential thyroid hormone space. If this was the only answer one would suspect that the time of maximal concentration would be the same as that observed for normals. While this factor may be quite important in the pituitary gland where the time of maximal uptake is the same for both groups, it does not seem to be applicable to the CNS where there is a delay in reaching the greatest concentration.

A further possibility for the increased time necessary to reach a maximal concentration in the CNS is that there may be a reduced rate of utilization of the incorporated hormone by the thyroidectomized animal. This may be associated with a decrease in the rate of deiodination in these tissues (Table 1). Such a decrease in the deiodination of the triiodothyronine might tend to permit a higher accumulation of the hormone within the tissue before the rate of deiodination exceeds the rate at which the labelled hormone is concentrated. Once this point is reached the deiodination appears to progress at a slower rate in the thyroidectomized animals (Fig. 4), and levels off after the fourth hour. In the normals the per cent of free iodide in the plasma continued to increase with time.

In the presentation of the data, it was noted in almost every part of the CNS and in the pituitary that there was a short lag in the rate of uptake which occurred in the second half-hour period. From the manner in which the uptake curves progress, it would appear that all organs are participating in an initial rapid clearing of triiodothyronine from the blood during the first $\frac{1}{2}$ hour (Figs. 1, 2, 3). This has also been noted for the adrenals (unpublished data) and liver (28). There then follows a decreased rate of concentration in the tissues. The time when the rate of concentration of radioactivity by the CNS again shows an increasing slope (1 hour after the injection) corresponds well with the time when the glucuronide-triiodothyronine complex formed in the liver starts to appear in the plasma in sizeable concentrations (15). The pathway for entry of this complex into

the blood appears to be in part through biliary excretion since the bile is very rich in this complex, followed by intestinal resorption (4). This complex is then slowly cleared from the plasma, being practically gone within two hours, when the levels of concentration in the tissues again start to fall. During the next two hours, this complex again starts to appear slowly in the plasma, becoming relatively prominent four hours from the beginning of the experiment. This corresponds to the time when the kidney has been shown to contain large amounts of the complex (29) and coincides with a period in which the rate of fall of CNS radioactivity levels off, as does the fall in plasma activity, possibly due to reentry into the vascular channels of a fresh supply of the conjugated form of triiodothyronine from the kidney. The fact that the levels of concentration do not rise is probably due to the very low levels of hormone with which one is dealing at this time. Since not even traces of the glucuronide complex are shown in the pituitary lobes, muscle (15) or CNS by chromatographic studies, it is concluded that it is only a transport form of the thyroid hormone which is rapidly disassociated to give up the free triiodothyronine which enters the tissue. From this discussion, it would appear that the CNS concentration of triiodothyronine is very readily affected by blood levels of either the free or conjugated form of the hormone.

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REFERENCES

1. Ford, D. H. and J. Gross: *Endocrinology* **62**: 416. 1958.
2. Ford, D. H. and J. Gross: *Endocrinology* (In press).
3. Taurog, A., G. W. Harris, W. Tong and J. L. Chaikoff: *Endocrinology* **59**: 34. 1956.
4. Ford, D. H., K. R. Corey and J. Gross: *Endocrinology* **61**: 426. 1957.
5. Fisher, R. A. and F. Yates: *Statistical Tables for Biological, Agricultural and Medical Research*, Oliver & Boyd, Ltd., Edinburgh, 1938.
6. Schultz, R. L., E. A. Maynard and D. C. Pease: *Am. J. Anat.* **100**: 369. 1957.
7. Maynard, E. A., R. L. Schultz and D. C. Pease: *Am. J. Anat.* **100**: 409. 1957.
8. Sloviter, H. A. and F. Morel: *Arch. Biochem.* **62**: 217. 1956.
9. Ford, D. H.: *Acta Anat.* (In press).
10. Ford, D. H. and S. Kantounis: *J. Comp. Neurol.* **108**: 91. 1957.
11. Flanigan, S., E. R. Gabrieli and P. D. MacLean: *Arch. Neurol. & Psych.* **77**: 588. 1957.
12. Ford, D. H. and A. Hirschman: *Anat. Rec.* **130**: 302. 1958.
13. Tata, J. R.: *Biochem. et Biophys. Acta.* **28**: 95. 1958.
14. Sensenbach, W., L. Madison, S. Eisenberg and L. Ochs: *J. Clin. Invest.* **33**: 1434. 1954.
15. Hoexter, F. M.: *Endocrinology* **54**: 1. 1954.
16. Barker, S. B.: *Physiol. Rev.* **31**: 205. 1951.
17. Fazekas, J. F., F. B. Graves and R. W. Alman: *Endocrinology* **48**: 169. 1951.

18. HAMMETT, F. S.: *J. Comp. Neurol.* **41:** 171. 1926.
19. EAYRS, J. T. AND S. H. TAYLOR: *J. Anat.* **85:** 350. 1951.
20. EAYRS, J. T.: *Nature* **172:** 403. 1953.
21. EAYRS, J. T.: *J. Anat.* **88:** 164. 1954.
22. EAYRS, J. T. AND G. HORN: *Anat. Rec.* **121:** 53. 1955.
23. BROWNING, T. B., R. W. ATKINS AND H. WEINER: *Arch. Int. Med.* **93:** 938. 1954.
24. CONDON, J. V., D. R. BECKER AND F. A. GIBBS: *J. Clin. Endocrinol.* **14:** 1511. 1954.
25. HORSTEN, G. P. M. AND J. TH. F. BOELES: *Arch. Int. Pharmodyn. et. Therap.* **78:** 93. 1949.
26. TIMIRAS, P. S., D. M. WOODBURY AND S. L. AGARWAL: *J. Pharmacol. and Exper. Therap.* **115:** 154. 1955.
27. TIMIRAS, P. S. AND D. M. WOODBURY: *Endocrinology* **58:** 181. 1956.
28. GROSS, J.: *Brookhaven Symposium in Biology, No. 7, "The Thyroid."* P. 107. 1955.
29. GROSS, J., D. H. FORD, S. SYMCHOWICZ AND J. HORTON: *In Ciba Found. Coll. Endocrinology* **10:** 182. 1957.

THE DESTRUCTION OF GLUCAGON BY THE BLOOD PLASMA FROM VARIOUS SPECIES[1]

I. ARTHUR MIRSKY, GLADYS PERISUTTI AND NEIL C. DAVIS

Department of Clinical Science, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Glucagon is hydrolyzed *in vitro* by blood plasma from various species. The highest rates occur with the plasmas from monkey and rat. Much lower rates of glucagonolysis are produced by plasmas from guinea-pig, chicken, man, rabbit, cow, dog and cat. The addition of streptokinase to the incubation mixture increases markedly the glucagonolytic activity of the plasmas from man, monkey and cat. In the presence of trace amounts of human plasma or of a crude euglobulin fraction of human plasma, streptokinase increases the glucagonolytic activity of the plasmas (or euglobulin fractions of such plasmas) from the various species. The data indicate that plasmin is responsible for the glucagonolytic action of plasma and that the activation of plasminogen by streptokinase is dependent upon a 'proactivator' which is present in human, monkey and cat plasmas, and absent or very low in the plasmas from the other species. It is proposed that the glucagonolytic action of native plasma is due to the release of endogenous activators of plasminogen and reflects a more general action of plasmin.

HUMAN plasma contains an enzyme system which catalyzes the destruction of adrenocorticotropin (1, 2, 3), somatotropin (3) and glucagon (3). In the presence of streptokinase, this hydrolytic activity of human plasma is increased markedly (3). The effect of streptokinase suggested that the active proteolytic enzyme, plasmin, formed from the conversion of the inactive precursor, plasminogen, is responsible for the destruction of these hormones. In accord is the marked acceleration of the destruction of the three hormones by a crude euglobulin fraction (plasminogen) of human plasma which occurs in the presence of streptokinase (3), the rapid rate of destruction produced by purified preparations of human plasmin (3) and the splitting of corticotropin A by bovine plasmin (4).

The blood plasma or serum from many infra-human species does not respond to amounts of streptokinase which readily produce an increase in the proteolytic or fibrinolytic activity of human plasma (5, 6, 7, 8, 9, 10,

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11, 12, 13, 14). In the presence of even minute amounts of euglobulin from human plasma, however, streptokinase catalyzes the conversion of rabbit, guinea-pig, dog, avian, and bovine plasminogen to plasmin (8, 9, 10, 13, 14, 15). Consequently, it has been proposed that the catalysis of the conversion of plasminogen to plasmin by streptokinase is dependent upon a co-factor or 'proactivator' which is presumed to be present in relatively large concentrations of human plasma but not in that of the dog, rabbit, guinea-pig, chicken, duck and rat (8, 13, 14). Accordingly, if plasmin is responsible for the destruction of some of the peptide hormones, the presence of streptokinase in amounts which are effective with human plasma should have relatively little or no effect on the proteolytic activity of the plasma from the above sub-human species. In the presence of the human plasma 'proactivator,' however, streptokinase should readily increase the proteolytic activity of the plasma. To test this thypothesis, the degradation of glucagon *in vitro* by fresh plasma from man, monkey, cow, dog, cat, rabbit, guinea-pig, rat and chicken was studied.

METHODS

Venous blood was drawn into heparinized syringes from men, monkeys, dogs, cats, chickens and cows. Cardiac blood was taken from rabbits, guinea-pigs and rats. In all instances the plasma was separated rapidly. The activity of the native and streptokinase-treated plasma was determined by incubating 1 ml. plasma with 0.5 ml. buffer containing 0.2 μmole of crystalline glucagon[2] (M.W. = 10,000) and 0.5 ml. MacIlvaine buffer (16) at pH 7.8 (without and with 1,000 units streptokinase). The streptokinase preparation (Varidase®)[3] assayed approximately 5,000 units per mg. The mixtures were incubated in test tubes in a Dubnoff shaker at 37° C for 90 minutes at which time the reaction was stopped by the addition of an equal volume of 10 per cent trichloracetic acid (TCA). Controls consisted of the same mixtures except that the plasma was added after the TCA. The glucagon comprising the substrate contained tracer quantities of I^{131} labeled glucagon prepared by the procedure described by Bauman *et al.* (17). The radioactivity of the whole TCA precipitated incubation mixture and of the TCA soluble portion were assayed with a well type scintillation counter and after appropriate corrections the amount of protein that was hydrolyzed was computed as described previously (18). An increase in the per cent of the total radioactivity which appears in the TCA filtrate indicates the hydrolysis of the substrate (3).

The effect of human 'proactivator' on the degradation of glucagon by streptokinase-treated plasma was determined by incubating 1 ml. plasma with 0.5 ml. buffer containing 0.2 μmole of crystalline glucagon and 0.5 ml. buffer at pH 7.8 containing 1,000 units streptokinase without and with 0.01 ml. human plasma. The human plasma was incubated with the streptokinase for 5 minutes before the addition of the other constituents of the incubation mixture. The action of the 0.01 ml. human plasma plus streptokinase was determined by incubating the same mixture except that the animal plasma was added after the precipitation with TCA.

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[3] We are indebted to Dr. J. M. Ruegsegger, Lederle Laboratories, American Cyanamid Company, for generous supplies of streptokinase (Varidase®).

Crude euglobulin (plasminogen) fractions of human and animal plasmas were prepared by precipitating fresh plasma with 19 volumes of distilled water and adjusting to pH 5.2 with 0.5 per cent acetic acid (19). The precipitate from each ml. of plasma was resuspended in 1 ml. of MacIlvaine's buffer at pH 7.8. Each incubation mixture consisted of 1 ml. of the animal plasma euglobulin solution, 0.5 ml. buffer containing 1,000 units streptokinase without and with 0.01 ml. human plasma euglobulin solution and 0.5 ml. buffer containing 0.2 μmole glucagon. The human plasma fraction was incubated with the streptokinase for 5 minutes before the addition of the other constituents of the incubation mixture. In addition, the activity of the human euglobulin fraction was determined by incubating the same mixture except for the animal plasma fraction which was added after the precipitation with TCA.

Radioautographs of chromatograms of various incubation mixtures were prepared by the procedure described by Tong, Taurog and Chaikoff (20).

RESULTS

Native plasma (0.5 ml. per ml. incubation mixture) from all the species studied destroyed glucagon (0.1 μmole per ml. incubation mixture) to some degree during the 90 minute incubation period. The data (mean ±S.E.) obtained with plasma from the various species is illustrated in Figure 1. The highest rate of glucagon degradation was produced with monkey plasma (14.2 ±1.5%). Untreated rat plasma also produced a rela-

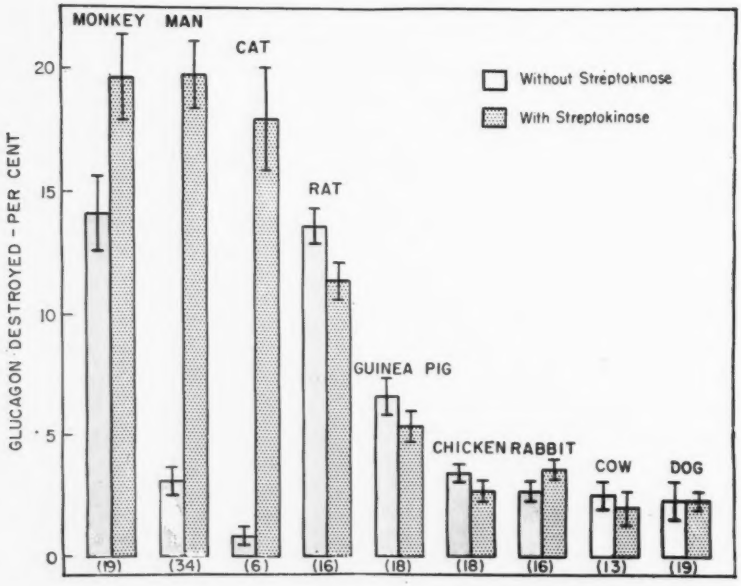

FIG. 1. Degradation of Glucagon by the Plasma from Various Species.

The per cent of glucagon (mean ±S.E.) destroyed in 90 minutes incubation with plasma, without and with streptokinase, was computed as indicated in text. The numerals refer to the number of samples that were studied.

tively high rate of glucagon destruction (13.7 ±0.7%). The mean activity of 34 samples of human plasma (3.1 ±0.6%) was lower than that observed earlier (3) and in the range of the activities obtained with the native plasmas from guinea-pig, rabbit, chicken, dog and cat.

The addition of streptokinase (1,000 units per ml. plasma) to the incubation mixture resulted in a marked increase in the rate of glucagon degradation produced by human, monkey and cat plasmas (Fig. 1). No statistically significant change in the activity of the plasma from any of the other species occurred in the presence of streptokinase. Radioautographs of chromatograms of aliquots of the incubation mixtures containing glucagon, plasma and streptokinase are illustrated in Figure 2. 20 λ of each incubation mixture was taken at 0, 1 and 2 hours and applied as a streak to the pages. Although it was necessary to overload the chromatograms to do so, the radioautographs reveal that some degree of glucagonolytic activity is exerted by the plasma from each of the species.

The influence exerted by the 'proactivator' present in human plasma is illustrated in Figure 3 which depicts the data obtained (mean ±S.E.) after incubation of the plasmas (0.5 ml. per ml. incubation mixture) with glucagon (0.1 μmole per ml. incubation mixture) and streptokinase (500 units per ml. incubation mixture) with and without human plasma (0.005 ml. per ml. incubation mixture). Appropriate corrections were made for the activity of the human plasma plus streptokinase. Only those species whose plasmas were not activated by streptokinase were studied. It is evident that the presence of the 'proactivator' in the relatively small volume of human plasma produced a marked acceleration in the rate of glucagon degradation by the plasma from the guinea-pig and the dog. The activity of rat plasma was moderately increased in the presence of the 'proactivator' while the activity of rabbit plasma was not changed significantly. The activity of the streptokinase-treated plasmas from chicken and cow was inhibited by the human plasma.

In order to reduce the quantity of plasmin inhibitors present in the human and animal plasmas, the effect of a euglobulin fraction prepared from fresh human plasma (equivalent to 0.005 ml. plasma per ml. incubation mixture) on the activity of a similar animal plasma fraction (equivalent to 0.5 ml. plasma per ml. incubation mixture) was studied after a preliminary incubation of the former with streptokinase (500 units per ml. incubation mixture). Appropriate corrections for the activity of the human plasma factor plus streptokinase were made. The data (mean ±S.E.) obtained are illustrated in Figure 3. As with the native plasma, the streptokinase-treated human euglobulin fraction increased the activity of the euglobulin fraction of the guinea-pig plasma and, to a lesser degree, of the dog plasma fraction. In addition, the streptokinase-treated human plasma fraction produced a more marked enhancement in the proteolytic activity of the rat and rabbit euglobulin plasma fractions than did an equivalent amount

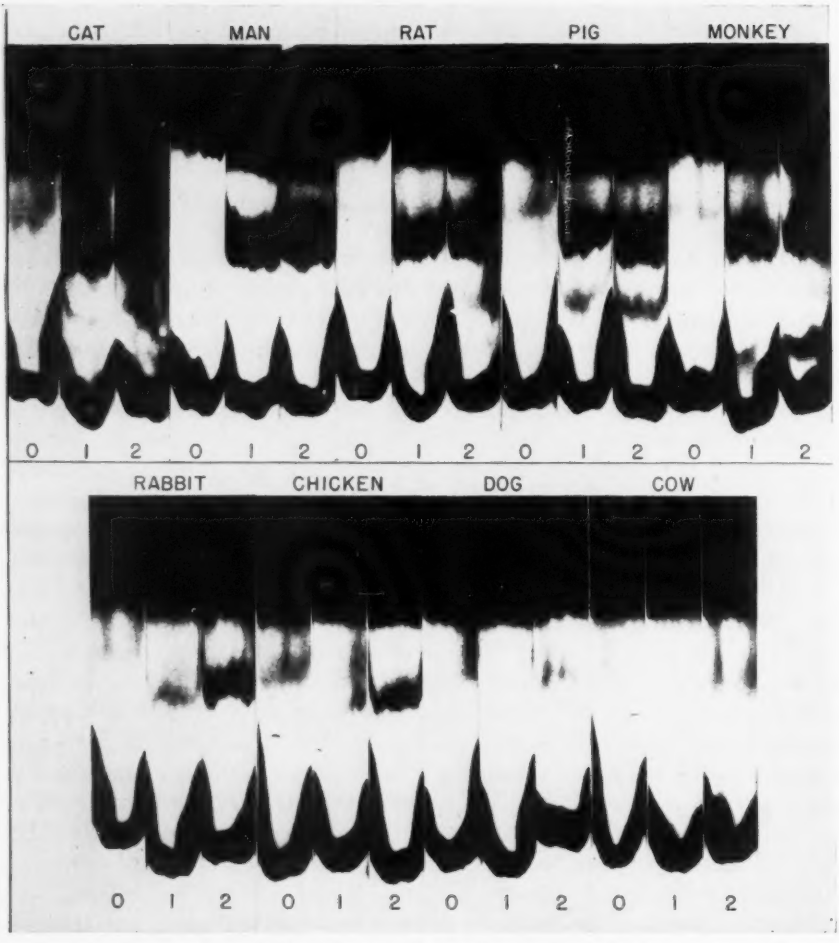

FIG. 2. Degradation of Glucagon by Streptokinase-Treated Plasma from Various Species.

Radioautographs of descending chromatograms of 20 λ aliquots of incubation mixtures removed at designated intervals (hours), streaked on paper and chromatographed as indicated in text.

of native human plasma. Further, whereas the native human plasma produced an inhibition in the activity of the plasma from chickens and cows, the euglobulin fraction of human plasma produced a marked increase in the activity of the bovine plasma fraction and a small but statistically significant increase in the activity of the chicken plasma fraction.

DISCUSSION

The data reported herein reveal that plasma from monkeys and rats can destroy glucagon at a relatively high rate while that from man, cat,

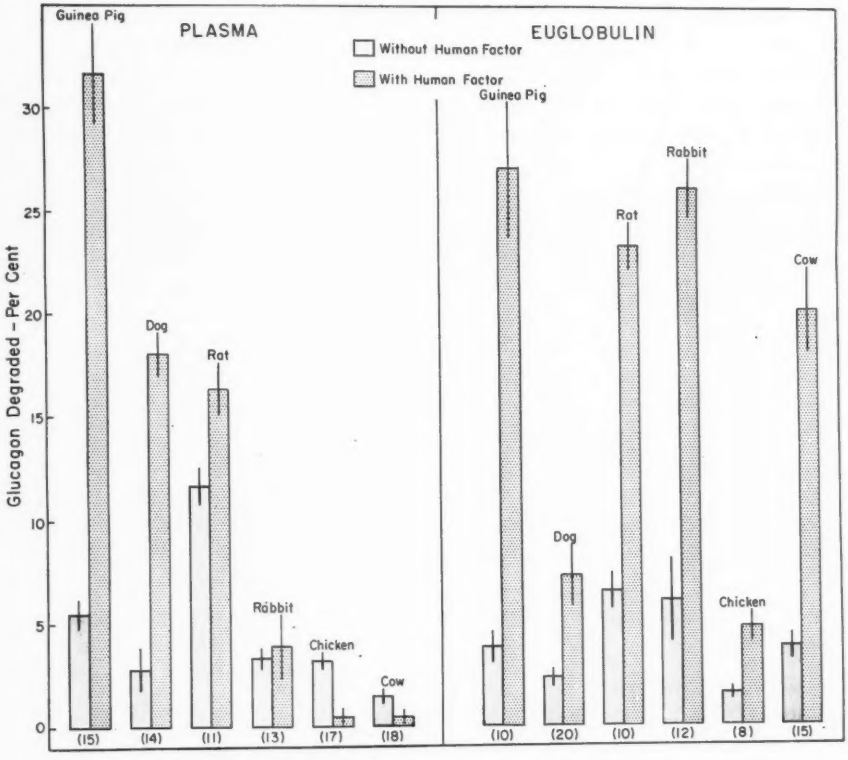

FIG. 3. The Effect of Human 'Proactivator' on the Glucagonolytic Action of Streptokinase-Treated Plasmas from Various Species.

The methods employed are indicated in text. The numerals refer to the number of plasmas studied.

guinea-pig, rabbit, chicken, dog and cow exerts a much lower rate of destruction. If it be assumed that 0.1 μg. glucagon is equivalent to one cat unit (the amount per kilogram of body weight which produces a rise of 30 mg. per cent in the blood sugar of the cat) (21), then the rates of destruction of glucagon by monkey and rat plasmas in 90 minutes are 56.8 ± 6.0 and 54.8 ± 2.8 cat units respectively. Even the much lower rates of hydrolysis produced by guinea-pig, chicken, human, rabbit, bovine, dog and cat plasmas are not negligible when expressed as cat units of glucagon hydrolyzed per ml. plasma per 90 minutes, viz., 25.4 ± 3.2, 13.6 ± 1.6, 12.4 ± 2.4, 10.8 ± 1.6, 10.0 ± 2.4, 9.2 ± 2.0, and 3.2 ± 1.6 units respectively.

That the hydrolysis of glucagon by the native plasma of man is due to plasmin is suggested by the marked increase in the glucagonolytic activity of the plasma in the presence of streptokinase. The absence of a significant affect of the same concentration of streptokinase on the glucagonolytic activity of the plasmas from the rat, guinea-pig, rabbit, chicken, dog and

cow is in accord with the reports that the plasminogen in the plasma of these species is relatively resistant to the action of streptokinase (5, 6, 7, 8, 9, 10, 11, 12, 13, 14).

The only sub-human species whose plasma developed an increased glucagonolytic activity in the presence of streptokinase are the monkey and cat. This observation suggests that whereas the plasmas of the other sub-human species do not contain some 'proactivator' essential to the action of streptokinase, monkey and cat plasmas do contain such a factor or are more susceptible to the action of streptokinase. In accord is the demonstration by Cliffton and Downie (6) that the caseinolytic activity of monkey plasma is increased by streptokinase. No data on the influence of streptokinase on the caseinolytic or fibrinolytic activity of cat plasma is available.

Although a concentration of streptokinase which readily catalyzes the conversion of human plasminogen to plasmin is ineffective in increasing the glucagonolytic activity of the plasmas from the various sub-human species other than monkey and cat, the addition of a small amount of human plasma together with the streptokinase accelerates the activity of guinea-pig, dog and rat plasmas. Under similar conditions, however, the activity of the plasmas from rabbits, chickens and cows is not increased. Since human and other plasmas contain plasmin inhibitors, the negative results may be due to the addition of such inhibitors which in turn mask the effect of streptokinase. In accord is the demonstration that in the presence of a crude euglobulin fraction which is relatively poor in inhibitors, streptokinase produces a marked increase in the activity of the crude euglobulin fraction of the plasmas from all the sub-human species.

The preceding is in accord with the hypothesis that a human 'proactivator' may be involved in the process whereby streptokinase catalyzes the conversion of plasminogen to plasmin. The demonstration that some activation of the plasminogens from the plasmas of various sub-human species can be produced by very high concentrations of streptokinase (22, 23) does not negate this hypothesis. Nor is the hypothesis rendered invalid by the studies which suggest the activator of sub-human plasminogens is a streptokinase-plasminogen complex (15, 23) or plasmin itself (10, 12, 24). It is pertinent to note, however, that irrespective of the manner whereby streptokinase catalyzes the conversion of plasminogen to plasmin, this conversion can be produced by a variety of other 'activators' (13, 24). Among such activators are factors which are found in nearly all the tissues of various species (13, 14).

An activator of plasminogen appears rapidly in the circulation of animals and men after exposure to electroshock (25, 26, 27), the injection of pyrogens (26, 27), and the production of a local ischemia (26, 27). The release of such an activator from the tissues into the circulation is believed

to be responsible for the increased fibrinolytic and/or proteolytic activity of the blood which is observed in such varied conditions as anaphylactic shock (28, 29), haemorrhagic, traumatic and thermal shock (30, 31, 32, 33, 34), acute febrile states (27, 35, 36), liver disease (37, 38, 39), toxemia of pregnancy (39), total body irradiation (40), electroshock convulsions (25, 27, 41), severe exercise (27, 42), local ischemia (27, 43), emotional tension (44, 45), after injections of adrenocorticotropin (32), adrenalin (27, 42, 46, 47), acetylcholine, 5-hydroxytryptamine, and Pituitrin® (46, 47).

The release of small amounts of endogenous activators of plasminogen may be responsible for the glucagonolytic action of plasma from apparently normal men and animals. It is doubtful that this contributes significantly to the rapid rate of destruction of glucagon by the intact animal (48, 49) or by liver extracts (50, 51, 52). Not only does the 'glucagonase' of liver extracts exhibit a more marked glucagonolytic activity but it differs from plasmin in its resistance to inhibitors of plasmin while inhibitors of 'glucagonase' do not affect the action of plasmin (53). The glucagonolytic action of plasma, however, may reflect some more general process involving plasmin since this enzyme catalyzes the hydrolysis of many proteins. Thus, in addition to its potent fibrinolytic action, plasmin catalyzes the hydrolysis of adrenocorticotropin (3, 4), somatotropin (3), serum complement (54), AC globulin (55), various plasma globulins (56), casein (57), fibrinogen (58) and other proteins. Although plasmin differs from trypsin in many respects, it resembles trypsin in its action on a number of proteins and on synthetic polypeptides containing the basic amino acids arginine and lysine (24).

The variety of situations which provoke the release of activators of plasminogen and the numerous proteins which are hydrolyzed by plasmin suggest some function in the sequence of events which characterize the somatic responses to noxious stimuli. In accord is the observation that the incubation of plasma or plasma globulins with plasmin results in the rapid production of polypeptides with vasodilator and myotrophic properties (59, 60, 61). These peptides appear to be identical with the bradykinin which is formed when plasma globulins are incubated with trypsin (61, 62). Other polypeptides which induce an increase in vascular permeability are formed under *in vitro* conditions which suggest that they are products of plasmin activity (61). The demonstration that the incubation of α-2-globulin with trypsin rapidly yields a specific tetradecapeptide which, in turn, is split by renin at its leucyl-leucine linkage to release the decapeptide angiotensin I (or hypertensin I) (63, 64) suggests that a similar sequence of events may be initiated by the action of plasmin. These and other considerations suggest the hypothesis that a variety of physiologically-active peptides are produced when plasminogen is activated in the circulation of

the animal exposed to noxious stimuli. The effectiveness of such activation, however, will be dependent upon the extent to which the plasmin is inhibited by circulating inhibitors.

REFERENCES

1. Reiss, M., F. F. Badrick, I. D. K. Halkerston and C. Plaice: *Nature* **168**: 206. 1951.
2. Pincus, G., T. F. Hopkins and O. Hechter: *Arch. Biochem. and Biophys.* **37**: 408. 1952.
3. Mirsky, I. A., G. Perisutti and N. C. Davis: *J. Clin. Invest.* **38**: 14. 1959.
4. White, W. F. and A. M. Gross: *J. Am. Chem. Soc.* **79**: 1141. 1957.
5. Tillet, W. J.: *Bact. Rev.* **2**: 161. 1938.
6. Cliffton, E. E. and G. R. Downie: *Proc. Soc. Exp. Biol. and Med.* **73**: 559. 1950.
7. Lewis, J. H. and J. H. Ferguson: *J. Clin. Invest.* **29**:1059. 1950.
8. Geiger, W. B.: *J. Immunol.* **69**: 597. 1952.
9. Mullertz, S. and M. Lassen: *Proc. Soc. Exp. Biol. and Med.* **82**: 264. 1953.
10. Sherry, S.: *J. Clin. Invest.* **33**: 1054. 1954.
11. Troll, W., S. Sherry and J. Wachman: *J. Biol. Chem.* **208**: 85. 1954.
12. Troll, W. and S. Sherry: *J. Biol. Chem.* **213**: 881. 1955.
13. Astrup, T.: *Blood* **11**: 781. 1956.
14. Mullertz, S.: *Ann. N. Y. Acad. Sci.* **68**: 38. 1957.
15. Ablondi, F. B. and J. J. Hagan: *Proc. Soc. Exp. Biol. and Med.* **95**: 195. 1957.
16. MacIlvaine, T. C.: *J. Biol. Chem.* **49**: 183. 1921.
17. Bauman, A., M. A. Rothschild, R. S. Yalow and S. A. Berson: *J. Clin. Invest.* **34**: 1359. 1955.
18. Mirsky, I. A., G. Perisutti and F. J. Dixon: *J. Biol. Chem.* **214**: 397. 1955.
19. Milstone, H.: *J. Immun.* **42**: 109. 1941.
20. Tong, W., A. Taurog and I. L. Chaikoff: *J. Biol. Chem.* **191**: 665. 1951.
21. Foa, P. P., G. Galansino and G. Pozza: *Rec. Prog. Hormone Res.* **13**: 473. 1957.
22. Sherry, S.: Personal communication.
23. Ablondi, F. B. and J. J. Hagan: Abstracts Am. Chem. Soc. 134th meeting, Sept. 1958.
24. Sherry, S. and N. Alkajaersig: *Thromb. Diath. Haem.* **1**: 264. 1957.
25. Mullertz, S.: *Proc. Soc. Exp. Biol. and Med.* **81**: 675. 1952.
26. Sherry, S., R. I. Lindemeyer, A. P. Fletcher and N. Alkjaersig: *J. Clin. Invest.* **37**: 931. 1958.
27. Sherry, S., A. P. Fletcher and N. Alkjaersig: "Symposium on Connective Tissue, Thrombosis and Atherosclerosis." Academic Press, N. Y. (In press.)
28. Imperati, L.: *Riv. Pat. Speriment.* **23**: 313. 1939.
29. Rocha e Silva, M., S. Andrade and R. M. Teixeira: *Nature* **157**: 801. 1946.
30. Tagnon, H. J., M. L. Levinson, C. S. Davidson and F. H. L. Taylor: *Am. J. Med. Sci.* **211**: 88. 1946.
31. Male, R. H.: *J. Path. and Bact.* **60**: 413. 1948.
32. Cliffton, E. E.: *J. Lab. and Clin. Med.* **39**: 105. 1952.
33. Westphal, U., W. Kocholaty, B. McDonald, E. King and H. Jensen: *Proc. Soc. Exp. Biol. and Med.* **75**: 862. 1950.
34. Macfarlane, R. G.: *Lancet* **232**: 10. 1937.
35. Ratnoff, O. D.: *Bull. Johns Hopkins Hosp.* **88**: 304. 1951.
36. Von Kaulla, K. N. and J. Weil: *Fed. Proc.* **15**: 194. 1956.
37. Goodpasture, E. W.: *Bull. Johns Hopkins Hosp.* **25**: 330. 1914.
38. Ratnoff, O. D.: *Bull. Johns Hopkins Hosp.* **83**: 29. 1949.

39. Smith, O. W. and G. V. S. Smith: *Science* **102:** 253. 1946.
40. Colgan, J., E. Gates and L. L. Miller: *J. Exp. Med.* **95:** 531. 1952.
41. Fantl, P. and S. E. Simon: *Aust. J. Exp. Biol. Med. Sci.* **26:** 521. 1948.
42. Biggs, R., R. G. Macfarlane and J. Pilling: *Lancet* **1:** 402. 1947.
43. Kwaan, H. C. and A. J. S. McFadzean: *Clin. Sci.* **15:** 245. 1956.
44. Macfarlane, R. G. and R. Biggs: *Lancet* **2:** 862. 1946.
45. Latner, A. L.: *Lancet* **1:** 194. 1947.
46. Kwaan, H. C., R. Lo and A. J. S. McFadzean: *Clin. Sci.* **16:** 241. 1957.
47. Lo, R. and A. J. S. McFadzean: *Clin. Sci.* **17:** 499. 1958.
48. Berson, S. A., R. S. Yalow and B. W. Volk: *J. Lab. and Clin. Med.* **49:** 331. 1957.
49. Narahara, H. T. and R. H. Williams: *Endocrinology* **60:** 285. 1957.
50. Snedecor, J. G. and W. B. MacGrath, Jr.: *Anat. Rec.* **113:** 588. 1952.
51. Vuylsteke, C. A. and C. de Duve: *J. Physiol.* (Paris) **47:** 304. 1955.
52. Mirsky, I. A. and G. Perisutti: *J. Biol. Chem.* **228:** 77. 1957.
53. Mirsky, I. A. and G. Perisutti: (Unpublished).
54. Pillemer, L., O. D. Ratnoff, L. Blum and I. H. Lepow: *J. Exp. Med.* **97:** 573. 1953.
55. Ferguson, J. H.: *Trans. 2nd Conf. on Blood Clotting and Allied Problems.* 1949.
56. Lewis, G. P.: *J. Physiol.* **140:** 285. 1958.
57. Remmert, LeM. and P. P. Cohen: *J. Biol. Chem.* **181:** 431. 1949.
58. Christensen, L. R.: *J. Genl. Physiol.* **28:** 363. 1945.
59. Beraldo, W.: *Am. J. Physiol.* **163:** 283. 1950.
60. Schachter, M.: *Br. J. Pharmacol.* **11:** 111. 1956.
61. Lewis, G. P.: *J. Physiol.* **140:** 285. 1958.
62. Rocha e Silva, M., W. T. Beraldo and G. Rosenfeld: *Am. J. Physiol.* **156:** 261. 1949.
63. Skeggs, L. T., Jr., K. E. Lentz, J. R. Kahn and N. P. Shumway: *J. Exp. Med.* **108:** 283. 1958.
64. Skeggs, L. T., Jr., J. R. Kahn, K. E. Lentz and N. P. Shumway: *J. Exp. Med.* **106:** 439. 1957.

THE EFFECTS OF THE ARYLSULFONYLUREAS ON THE RESPIRATORY ACTIVITY AND GLUCOSE METABOLISM OF ISOLATED RABBIT KIDNEY CORTEX[1]

NORBERT FREINKEL AND SIDNEY H. INGBAR

Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts, and the Howard Hughes Medical Institute

ABSTRACT

Slices of kidney cortex from fed and from fasted rabbits were incubated in the Warburg apparatus in glucose-free and glucose-containing phosphate-buffered media. The addition of 5×10^{-3} M Orinase *in vitro* effected an increase in cellular hydration and a profound reduction in oxygen consumption. Net disappearance of stable and radioactive glucose from the suspending medium was increased in five of six experiments. In all studies with 5×10^{-3} M Orinase, significantly smaller proportions of the assimilated glucose-C^{14} were incorporated into tissue lipids or recovered as radioactive CO_2. Under similar conditions, the metabolic product of Orinase, p-carboxyphenylsulfonylurea, was less than one-fifth as potent an inhibitor of the respiratory metabolism of surviving rabbit kidney cortex.

DESPITE extensive investigations designed to elucidate the mechanism of action of the hypoglycemic arylsulfonylureas, the data concerning their effects upon the respiratory metabolism of isolated cellular preparations are meager. In the present studies, surviving slices of rabbit kidney cortex were incubated in the presence of 1-butyl-3-p-tolylsulfonylurea (Orinase),[2] and 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea);[2] the principal metabolic end-product of Orinase in man (1, 2). The selection of kidney tissue as the experimental preparation was prompted by several considerations. First, since Orinase and its metabolites are excreted chiefly by the kidney (3), it might be anticipated that concentrations of drug in excess of plasma levels could occur in this

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[2] Orinase is the Upjohn trademark for its brand of 1-butyl-3-p-tolylsulfonylurea. Supplies of Orinase and 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea) used in these investigations were made available through the generosity of Dr. C. J. O'Donovan of Upjohn Company, Kalamazoo, Michigan.

area. Secondly, since many of the glycogenolytic, gluconeogenetic, and glucose-releasing systems which have been shown to be affected by Orinase in the liver (4, 5, 6) are also present in the kidney (7, 8, 9), it was felt that kidney tissue might afford another system for analysis of these phenomena. Finally, slices of rabbit kidney cortex constitute a hardy and metabolically-active preparation in which there is ready utilization of exogenous substrates, and in which the effects of metabolic inhibitors *in vitro* have been amply documented (10, 11).

METHODS

Seven experiments were performed. For each experiment, a male 2–3 Kg. rabbit was killed by a blow on the head. Slices of 0.3–0.4 mm. thickness were prepared from excised wedges of kidney cortex and floated on chilled saline. Occluded blood elements and plasma were removed from the slices by three successive five-minute washings with chilled saline. Slices were weighed on a torsion balance and introduced into 2.7 cc. of suspending medium contained within the main compartment of chilled Warburg vessels. The medium was composed of 0.131 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$ and 10 mM sodium phosphate buffer (pH 7.4). Experimental media were supplemented with the desired concentrations of the sodium salts of Orinase[2] or p-carboxyphenylsulfonylurea[2] and adjusted to pH 7.4 prior to the addition of phosphate buffer. The osmolarity of all media was maintained constant by appropriate reductions in the NaCl. Three separate lots of Orinase were employed: lot #11, 584-2 for experiments #1, 4, and 5; lots #11, 745-4 for experiments #2 and 3; lot #11, 715-12 for experiments #6 and 7. Only a single preparation of p-carboxyphenylsulfonylurea was available for examination. Center wells of the Warburg vessels were filled with 0.2 cc. 5% KOH and fluted strips of filter paper. Details of incubation and manometric techniques have been described previously (12). Ten minutes were allowed for gassing of flasks with pure O_2 and for thermal equilibration at 38° C. Thereafter, there was introduced from the sidearms 0.3 cc. of suspending medium containing 0.6 to 2.0 μc. of uniformly-labeled glucose-C^{14}[3] and sufficient unlabeled glucose to achieve a final glucose concentration of 196–232 mg.% in the main compartment. Following variable periods of incubation, slices were removed, weighed on a torsion balance, and quickly frozen with solid CO_2. The frozen tissues were employed for subsequent fractionation. In experiments in which the water-content of the slices was estimated, the wet slices were introduced into tared weighing bottles; wet-weight was determined immediately, and final dry-weight was measured following desiccation to constant weight in an oven at 105° C.

Aliquots of the suspending medium were analyzed for glucose by the Nelson modification of the Somogyi method (13) and precipitated as the glucosazone for radioactive assay. Center wells were rinsed and filter-paper strips were eluted with CO_2-free water. The alkaline washings and corresponding eluates were pooled. $C^{14}O_2$ was quantitatively precipiated as $BaC^{14}O_3$ with $BaCl_2$ and plated on stainless steel planchets. Extracts of total lipids were prepared by homogenizing the frozen slices with chloroform: methanol according to the method of Folch, Lees, and Sloane-Stanley (14). The extracts were washed twice to remove water-soluble contaminants and then evaporated to dryness in tared weighing vessels. The dried residues were resuspended in petroleum ether, and

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[3] Uniformly-labeled glucose-C^{14} was prepared by Tracerlab, Inc., Boston Massachusetts. The specific activity of this preparation was 0.274 mc./mM. Homogeneity was verified in the authors' laboratory by one-dimensional paper chromatography in butanol /glacial acetic acid/H_2O and propanol/ethyl acetate/H_2O solvent systems.

plated on lens paper discs contained within stainless steel planchets. All determinations were performed with duplicate vessels.

A Robinson windowless proportional flow counter was employed for radioactive assays and samples were corrected for self-absorption to a mass of 5 mg. Sufficient counts were observed to reduce the probable error of the measurement to less than 3%.

Results were expressed as follows: Oxygen consumption was estimated in terms of QO_2 (cu. mm. O_2 per hour per mg. of initial wet weight). Relative changes in the mass of the suspended slices during incubation were assessed by ratios of final/initial wet weights. The net assimilation of glucose, expressed as "μg./mg. initial wet weight," was derived on the basis of chemical estimates of the glucose concentration of the media at the beginning and the end of incubation, and a known volume of 3 cc. of suspending medium within each flask. Specific activities of the glucose in the final suspending media were determined on the basis of glucosazones and calculated as "counts/mg. glucose." The formation of radioactive CO_2 and lipids from uniformly-labeled glucose was quantitated in terms of "% of the total added radioactivity."

RESULTS

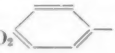
1) *Oxygen Consumption:* Results of the seven experiments are listed in Table 1. Inclusion of 5×10^{-3} M Orinase in the suspending medium effected a uniform and highly significant reduction in the oxygen consumption of surviving rabbit kidney slices. During the first hour of incubation in glucose-containing media, the QO_2 values of slices from fasted and from fed rabbits averaged 49.7% and 61.9% respectively of control values. Percentile inhibitions were even greater during the second and third hours of incubation. A comparable depression of respiratory activity by 5×10^{-3} M Orinase was observed in one experiment (#5) wherein glucose was omitted from the suspending medium. The metabolic product of Orinase, p-carboxyphenylsulfonylurea, was not as potent an inhibitor of respiratory metabolism. In three experiments, the average oxygen consumption during the first hour of incubation with $5\times10^{-}$ M p-carboxyphenylsulfonylurea was 91% of the control value. The inhibition approximated that which was observed with 1×10^{-3} M concentrations of Orinase.

2) *Slice Weight:* A significant reduction in the wet weight of slices occurred during incubation. The final wet weights of slices from control vessels in seven experiments averaged 78.8 of the initial wet weight (Table 1). Inclusion of 1×10^{-3} M Orinase or 5×10^{-3} M p-carboxyphenylsulfonylurea in the suspending medium did not affect the manifest loss of weight. However, a preservation of wet-tissue weight, and, in two instances, an actual gain in weight occurred during incubation with 5×10^{-3} M Orinase. Results of desiccation revealed that the apparent maintenance of wet weight by 5×10^{-3} M Orinase was caused by an increase in the tissue content of water (Table 2).

3) *Utilization of Glucose:* Based on chemical determinations, the net disappearance of glucose from the medium (i.e. μg. glucose/mg. initial wet weight) was increased by 5×10^{-3} M Orinase in five of six experiments (Table 3). Less pronounced effects were also produced by 1×10^{-3} M

TABLE 1. THE EFFECTS OF THE ARYLSULFONYLUREAS UPON THE RESPIRATORY METABOLISM OF SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt. #	Diet	Initial medium glucose (mg.%)	Initial slice wt. (mg.)	Incubat. (mins.)	Drug*	Conc. (1×10^{-3} M)	QO_2†	Final wt. / Initial wt.
1	Fasted	232	200 ± 10	90	None	—	1.63	0.767
					R-CH_3	5	0.96	0.928
					R-CH_3	1	1.22	0.750
					R-CH_3	0.1	1.42	0.792
2	Fasted	212	350 ± 10	120	None	—	1.71	0.765
					R-CH_3	5	0.74	0.962
3	Fasted	196	300 ± 10	150	None	—	2.02	0.758
					R-CH_3	5	0.95	0.967
					R-COOH	5	1.83	0.722
4	Fed	221	150 ± 10	90	None	—	1.69	0.817
					R-CH_3	5	0.84	0.946
5	Fed	0	150 ± 10	90	None	—	1.40	0.755
					R-CH_3	5	0.46	0.875
6	Fed	217	300 ± 10	180	None	—	1.80	0.838
					R-CH_3	5	1.17	1.114
					R-CH_3	1	1.52	0.826
					R-COOH	5	1.58	0.838
7	Fed	198	310 ± 10	180	None	—	1.68	0.818
					R-CH_3	5	1.20	1.105
					R-CH_3	1	1.46	0.778
					R-COOH	5	1.59	0.754

* R = $CH_3CH_2CH_2CH_2NHCONHSO_2$– 

R-CH_3 refers to 1-butyl-3-p-tolylsulfonylurea (Orinase) and R-COOH refers to 1-butyl-3-p-carboxyphenylsulfonylurea (p-carboxyphenylsulfonylurea).

† Average values for QO_2 (μl. of O_2/mg. initial wet weight/hr.) during the first hour of incubation.

TABLE 2. THE EFFECT OF THE ARYLSULFONYLUREAS UPON THE WATER-CONTENT OF SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt.	Drug	Conc. (1×10^{-3}M)	Slice weight (mg.)			Final water content (mg.)
			Initial	Final		
			wet	wet	dry	
3A	None	—	306.5	216.5	44.1	172.4
	R-CH_3	5	296.5	257.8	39.3	218.5
	R-COOH	5	307.5	214.3	46.0	168.3
7A	None	—	304.5	235.5	56.8	178.7
	R-CH_3	5	300.5	319.2	52.7	266.5
	R-CH_3	1	308.0	235.0	54.2	180.8
	R-COOH	5	309.5	231.2	53.2	178.0

Orinase and 5×10^{-3} M p-carboxyphenylsulfonylurea. Recalculation of results in terms of μg. glucose/mg. final wet weight reduced the magnitude but did not alter the direction of the differences between control and experimental values.

In preliminary studies with homogenates of rabbit kidney cortex, the presence of glucose-6-phosphatase activity was easily demonstrated (15). Enzyme activity per gram of tissue was increased in fasted animals. Similar dietary dependencies have recently been reported for the glucose-6-

TABLE 3. THE EFFECTS OF THE ARYLSULFONYLUREAS UPON THE METABOLISM OF GLUCOSE BY SURVIVING SLICES OF RABBIT KIDNEY CORTEX

Expt.* #	Drug	Conc. (1×10^{-3}M)	Net assimilation of glucose (μg./mg.)	Final S.A. glucose C^{14} (counts/mg.)†	% added C^{14} recovered as	
					Lipid	CO_2
1	None	—	6.04	67,191	0.21	5.3
	R-CH_3	5	8.34	73,413	0.12	4.6
	R-CH_3	1	7.60	66,702	0.23	5.5
	R-CH_3	0.1	6.07	70,551	0.24	4.9
2	None	—	6.32	56,100	0.56	9.7
	R-CH_3	5	5.39	56,015	0.29	4.4
3	None	—	5.59	24,813	0.30	6.3
	R-CH_3	5	6.40	21,812	0.18	5.3
	R-COOH	5	6.07	24,224	0.31	6.6
4	None	—	6.26	60,715	0.17	7.7
	R-CH_3	5	7.70	57,202	0.13	5.1
6	None	—	5.72	62,437	0.28	7.0
	R-CH_3	5	8.47	58,886	0.19	6.7
	R-CH_3	1	8.23	57,376	0.31	8.8
	R-COOH	5	7.84	61,286	0.31	6.4
7‡	None	—	8.57			
	R-CH_3	5	11.05			
	R-CH_3	1	10.94			
	R-COOH	5	10.45			

* Numbers refer to the same experiments for which partial results are tabulated on Table 1.

† S.A. = Specific activity of glucose in suspending medium at end of incubation.

‡ Radioactive glucose was not employed in Expt. #7.

phosphatase of rat kidney (9). However, despite the enzymatic potential for the release of glucose, and in contradistinction to the published findings for surviving rat tissue (8), it has not been possible to demonstrate a measurable production of glucose during the prolonged incubation of kidney slices from fed or fasted rabbits in simple saline media (15). Nonetheless, it remained possible that such phenomena could occur in the glucose-containing systems employed in the present studies. Therefore, the specific radioactivities of the glucose in the media were examined at the end of incubation in order to assess the possibility that the manifest effects of Orinase resulted from an inhibition of glucose production. Within the limits of analytical accuracy, the final specific activities of the control

and Orinase-containing media were not significantly different (Table 3). Hence, the apparent increase in the assimilation of glucose in the presence of Orinase cannot be attributed to a depression of glucose production.

4) *The Metabolism of Glucose-C^{14}:* In the five experiments where radioactive measurements were obtained, the absolute incorporation of glucose-C^{14} into lipids was reduced an average of 39.5% below control values by 5×10^{-3} M Orinase (Table 3). Comparable effects were not obtained with 5×10^{-3} M p-carboxyphenylsulfonylurea. The inhibitory action of Orinase paralleled the depression of QO_2. The percentage of the added C^{14} which was evolved as radioactive CO_2 was also consistently reduced by 5×10^{-3} M Orinase. However, the effects were modest in those experiments in which Orinase increased the net assimilation of glucose.

To document the metabolic disposition of this glucose, the isotopic data were recalculated in terms of the percentile distribution of the radioactive glucose which disappeared from the medium (i.e. percentage of assimilated glucose C^{14}). This calculation disclosed that a significantly smaller proportion of the assimilated glucose was oxidatively decarboxylated or incorporated into lipids in the experiments where 5×10^{-3} M Orinase enhanced the manifest removal of glucose from the medium.

DISCUSSION

During the incubation of surviving slices of rabbit kidney cortex in phosphate-buffered saline and an atmosphere of oxygen, 5×10^{-3} M Orinase effected an approximate halving of oxygen consumption. At the same time, cellular water increased, an effect which has been previously described as an invariable concomitant of respiratory inhibition in isolated rabbit kidney (11). These changes in oxidative activity and cellular hydration occurred in glucose-free as well as glucose-supplemented media. Glucose-C^{14} was employed to document some of the metabolic sequelae. In all studies, the absolute incorporation of radioactivity into tissue lipids was profoundly reduced by 5×10^{-3} M Orinase. However, effects upon the utilization of glucose were more variable. In five of six experiments, 5×10^{-3} M Orinase caused a moderate apparent increase in the net disappearance of glucose from the suspending medium. Measurements of the final specific activities of the uniformly-labeled glucose in the suspending media would make it appear unlikely that this reflected merely an inhibition of renal glucose-production. An alteration in the metabolic disposition of the glucose was indicated by a uniform reduction in the percentage of the assimilated counts which appeared as radioactive CO_2 or lipid. From the data it may be inferred that an increased accumulation of such glycolytic intermediates as lactic acid must have occurred. The less pronounced effects of Orinase upon the absolute than upon the proportional production of radioactive CO_2 would also be consonant with a relative preservation of glycolytic pathways and a continued elaboration of

CO_2 from sources other than the tricarboxylic acid cycle, such as, possibly, the renal phosphogluconate oxidation pathway (16). These phenomena were not related to previous dietary intake since tissues from fasted as well as from fed donor animals were inhibited to a similar extent. Three separate lots of crystalline Orinase were employed for the *in vitro* observations. It would thus seem unlikely that the results can be attributed to a contaminant which is not present in the commercial, and clinically efficacious, preparations of the drug.

The effects upon oxidative metabolism were closely linked to chemical structure. Mudge has reported that the hydration and QO_2 of surviving-rabbit kidney cortex is unaffected by the substituted sulfonamides which inhibit carbonic anhydrase (11). In the present studies, p-carboxyphenylsulfonylurea, which differs from Orinase only by the substitution of a —COOH for the terminal $—CH_3$ group (1, 2), was less than one-fifth as potent as Orinase in depressing oxygen consumption. Although the differences between Orinase and p-carboxyphenylsulfonylurea may have resulted from variation in cellular penetration or fixation, it is of interest that the latter compound does not produce hypoglycemia in man (17).

Whether the above observations bear any relevance to the clinical action of Orinase remains to be defined. There is some precedent for the induction of hypoglycemia *in vivo* by a generalized depression of cellular respiration. Recently, Steiner and Williams have shown that the biguanide derivatives reduced blood sugar by an inhibition of electron transport at the cytochrome level (18). However, the biguanide derivatives are effective in types of diabetes which do not usually respond to Orinase (19), (cf. pancreatectomized animals; "brittle juvenile human diabetes") and, hence, it does not seem likely that the effects of Orinase are mediated in the same way. This does not preclude the possibility that Orinase may be selectively concentrated in certain organs or subcellular sites and there induce a localized histotoxic anoxia. If such were the case, the effects of Orinase to a) release performed stores of insulin (20, 21, 22), or b) retard the degradation of insulin (23), or c) diminish hepatic production of glucose (24, 4, 5, 6) could be secondary to a localized change in respiratory metabolism. It is even conceivable that a localized "Pasteur effect" induced by Orinase could cause focal changes in glucose utilization. Alternatively, the present findings may constitute an isolated toxic phenomenon which is unrelated to the therapeutic action of Orinase. The available data do not justify attempts to differentiate between these possible interpretations. It should be stressed that only one tissue from a single species was examined, and that the experimental concentrations of drug were five to ten times as great as those plasma levels which are therapeutically effective *in vivo*. Clearly, at present, more than descriptive analysis is not warranted.

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REFERENCES

1. Louis, L. H., S. S. Fajans, J. W. Conn, W. A. Struck, J. B. Wright and J. L. Johnson: *J. Am. Chem. Soc.* **78:** 5701. 1956.
2. Dorfmueller, T.: *Dtsch. Med. Wchschr.* **81:** 888. 1956.
3. Forist, A. A. and T. Chulski: *Metabolism* **5:** 807. 1956.
4. Vaughn, M.: *Science* **123:** 885. 1956.
5. Tyberghein, J. M., Y. D. Halsey and R. H. Williams: *Proc. Soc. Exper. Biol. & Med.* **92:** 322. 1956.
6. Ashmore, J., G. F. Cahill, Jr., and A. B. Hastings: *Metabolism* **5:** 774. 1956.
7. Goda, T.: *Biochem. Zeitschr.* **297:** 134. 1938.
8. Teng, C. T.: *Arch. Biochem. Biophys.* **48:** 409 and 415. 1954.
9. Froesch, E. R., J. Ashmore and A. E. Renold: *Endocrinology* **62:** 614. 1958.
10. Mudge, G. H.: *Amer. J. Physiol.* **165:** 113. 1951.
11. Mudge, G. H.: *Amer. J. Physiol.* **167:** 206. 1951.
12. Freinkel, N. and S. H. Ingbar: *J. Clin. Endocrinol. & Metab.* **15:** 442. 1955.
13. Nelson, N.: *J. Biol. Chem.* **153:** 375. 1944.
14. Folch, J., M. Lees and G. H. Sloane-Stanley: *Fed. Proc.* **13:** 209. 1954.
15. Freinkel, N.: Unpublished observations.
16. Bloom, B. and D. Stetten, Jr.: *J. Amer. Chem. Soc.* **75:** 5446. 1953.
17. Fajans, S. S., L. H. Louis, H. S. Seltzer, R. D. Johnson, R. D. Gittler, A. R. Hennes, B. L. Wajchenberg, I. P. Ackerman and J. W. Conn: *Metabolism* **5:** 820. 1956.
18. Steiner, D. F. and R. H. Williams: *Clin. Res.* **6:** 55. 1958.
19. Williams, R. H., J. M. Tyberghein, P. M. Hyde and R. L. Nielsen: *Metabolism* **6:** 311. 1957.
20. Loubatières, A.: *Presse Méd.* **63:** 1701. 1955.
21. Colwell, A. R., Jr., J. A. Colwell and A. R. Colwell, Sr.: *Metabolism* **5:** 749. 1956.
22. Pozza, G., G. Galansino and P. P. Foà: *Proc. Soc. Exp. Biol. & Med.* **93:** 539. 1956.
23. Mirsky, I. A., G. Perisutti and D. Diengott: *Metabolism* **5:** 156. 1956.
24. Moorhouse, J. A. and R. M. Kark: *Metabolism* **5:** 847. 1956.

A MYOTROPHIC AGENT AND GONADOTROPHIN INHIBITOR, 19-NORTESTOSTERONE-17-BENZOATE

LEONARD J. LERNER,[1] FRED J. HOLTHAUS, JR. AND CHARLES R. THOMPSON[2]

Scientific Division, The Wm. S. Merrell Co., Cincinnati, Ohio

ABSTRACT

19-Nortestosterone-17-benzoate, a new ester of 19-nortestosterone, has been compared with testosterone propionate for several biological activities. Testosterone propionate and 19-nortestosterone-17-benzoate are myotrophic and suppress production or release of pituitary gonadotrophins. Unlike testosterone propionate, the 19-nortestosterone derivative elicits these responses in doses which do not stimulate growth of seminal vesicles and ventral prostate in rats. The uterotrophic, anti-estrogenic and progestational potencies of the 2 androgens are similar. 19-Nortestosterone-17-benzoate is devoid of estrogenic ativity. A 6-week study in intact and castrate male and female rats demonstrate that the biologic properties of 19-nortestosterone-17-benzoate are typical of those of an androgen. This steroid was orally inactive at the dose tested.

ANDROGENIC steroids in the male, in addition to stimulating primary and secondary sex organs, are protein anabolic (1) and inhibit production of pituitary gonadotrophins (2, 3). Eisenberg and Gordan (1) demonstrated that trophic activity of androgens on the levator ani muscle of rats is a measure of protein anabolism. 19-Nortestosterone and derivatives of 19-nortestosterone have been reported to differ from testosterone propionate by being myotrophic in doses which produced no stimulation of the reproductive organs (4–7). There has been little reported on the antigonadotrophin activity of 19-nortestosterone derivatives.

In the present investigation, the biologic activities of 19-nortestosterone-17-benzoate,[3] a new ester of 19-nortestosterone, have been compared to those of testosterone propionate.

MATERIALS AND METHODS

Compounds were administered in solution or suspension in olive oil.

Myotrophic and androgenic assays. The method of Eisenberg and Gordan (1), as modified by Hershberger, Shipley, and Meyer (4), was employed. Weanling male rats weighing between 40 and 50 gm. were castrated and divided into dose groups of 5 or 10 animals each. Starting on the day following castration, the compounds were adminis-

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[1] Present address: The Squibb Institute for Medical Research, New Brunswick, New Jersey.

[2] Present address: Cutter Laboratories, Berkeley, California.

[3] Obtained from Organics, Inc., Chicago. Illinois.

tered once a day for 7 days. The animals were sacrificed by decapitation on the following day and seminal vesicles, ventral prostate and levator ani muscles removed and weighed. Fluid was expressed from the seminal vesicles prior to weighing.

Myotrophic and androgenic activities of 19-nortestosterone-17-benzoate and testosterone propionate were also determined in immature intact rats. The techniques employed in the intact rats were identical to those described for the castrate rats.

Uterotrophic and anti-estrogenic activities. Uterotrophic and anti-estrogenic activities were determined in 6–8 gm. immature female mice. 19-Nortestosterone-17-benzoate or testosterone propionate was administered subcutaneously twice a day for 3 days to dose groups of 5 or 10 animals, either alone or in combination with 0.3 μg. of estradiol benzoate. The animals were sacrificed on the day following the last injection. The amounts of fluid in the uteri were estimated, the uteri removed, fluid expressed, and the uteri weighed.

Estrogenic activity. The vaginal smear technique in castrate rats was employed. 19-Nortestosterone-17-benzoate was administered subcutaneously in 2 doses 8 hours apart to dose groups of 10 rats each. Vaginal smears were obtained 56, 64, and 72 hours after the first dose.

Anti-gonadotrophic activity. Gonadotrophin inhibition was measured by the parabiotic rat method of Byrnes and Meyer (8). Thirty-day-old female litter mate rats were joined in parabiosis and the right partner was ovariectomized. The compounds were administered once a day for 10 days to the castrated partner, beginning on the day of operation. The animals were sacrificed on the 11th day, the ovaries and uteri were removed and weighed.

Anti-gonadotrophic activity was also measured in immature male rats. The compounds were administered subcutaneously once daily for 10 days. The animals were sacrificed on the day following the last injection and testes, seminal vesicles, ventral prostates and levator ani were removed and weighed. The fluid was expressed from the seminal vesicles before weighing.

Progestational activity. Progestational activity was determined by the method of McPhail (9). Dose groups of 6 immature female rabbits each were administered 3 intramuscular injections of 5 μg. estradiol on alternate days. They then received 5 daily intramuscular injections of progesterone, testosterone propionate or 19-nortestosterone-17-benzoate. The rabbits were sacrificed on the day following the last injection. The uteri were weighed, sectioned and graded for degree of progestational response.

Subchronic study of 19-nortestosterone-17-benzoate. Six groups of 10 male rats and 6 groups of 10 female rats, weighing 125–150 gm. each were employed. Of these, three male groups and three female groups were castrated approximately 10 days before starting the experiment.

Two groups of castrate and intact males and females were administered 19-nortestosterone-17-benzoate subcutaneously 5 days a week for 6 weeks, in daily doses of 3 mg./Kg. and 15 mg./Kg. Similar groups served as controls and were administered olive oil subcutaneously.

RESULTS

Myotrophic and androgenic activities. Myotrophic and androgenic activities of 19-nortestosterone-17-benzoate and testosterone propionate in castrate and in intact rats are summarized in Figure 1. The levator ani of intact and castrate rats increased in weight with smaller doses of 19-nortestosterone-17-benzoate than did either ventral prostate or seminal vesicles. The smallest dose of testosterone propionate required to produce an increase in levator ani weight also produced an increase in weight of

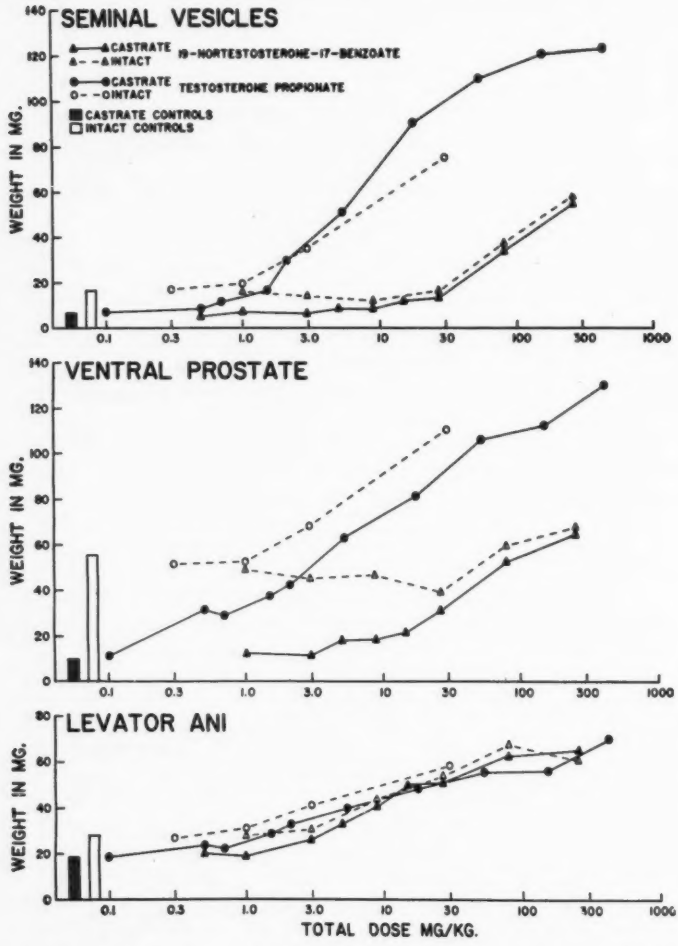

FIG. 1. Myotrophic and androgenic activity of 19-nortestosterone-17-benzoate and testosterone propionate in castrate and intact rats.

ventral prostate and seminal vesicles of intact rats and ventral prostate of castrate rats.

The dose of 19-nortestosterone-17-benzoate required to increase levator ani weight by 50% for castrate and intact animals is respectively 2 and 3 times that for testosterone propionate. The dose required to increase by 50% the weights of the seminal vesicles and ventral prostate for 19-nortestosterone-17-benzoate was much larger than that of testosterone propionate. The values ranged from 18 to 42 times that for testosterone propionate (Table 1). Oral doses of 50 mg/Kg. were ineffective in producing weight changes in levator ani and accessory sex organs.

TABLE 1. MYOTROPHIC AND ANDROGENIC DOSES (MG./KG.) IN CASTRATE AND INTACT MALE RATS PRODUCING 50% INCREASE IN ORGAN WEIGHT

Compound	Seminal vesicles		Ventral prostate		Levator ani	
	Castrate	Intact	Castrate	Intact	Castrate	Intact
19-nortestosterone-17-benzoate	13	40	4	270	2.5	8.1
Testosterone Propionate	0.7	1.4	0.12	6.4	1.2	2.8

Uterotrophic and anti-estrogenic activities. Testosterone propionate and 19-nortestosterone-17-benzoate had similar uterotrophic and estrogenic antagonism potencies in the immature mouse (Fig. 2).

Estrogenic activity. 19-Nortestosterone-17-benzoate was not estrogenic in doses ranging from 0.01 mg. to 5 mg.

Anti-gonadotrophic activity. The minimal dose for pituitary gonadotrophin suppression in the parabiotic rats was <0.01 mg. for testosterone propionate and was <0.1 mg. for 19-nortestosterone-17-benzoate (Table 2).

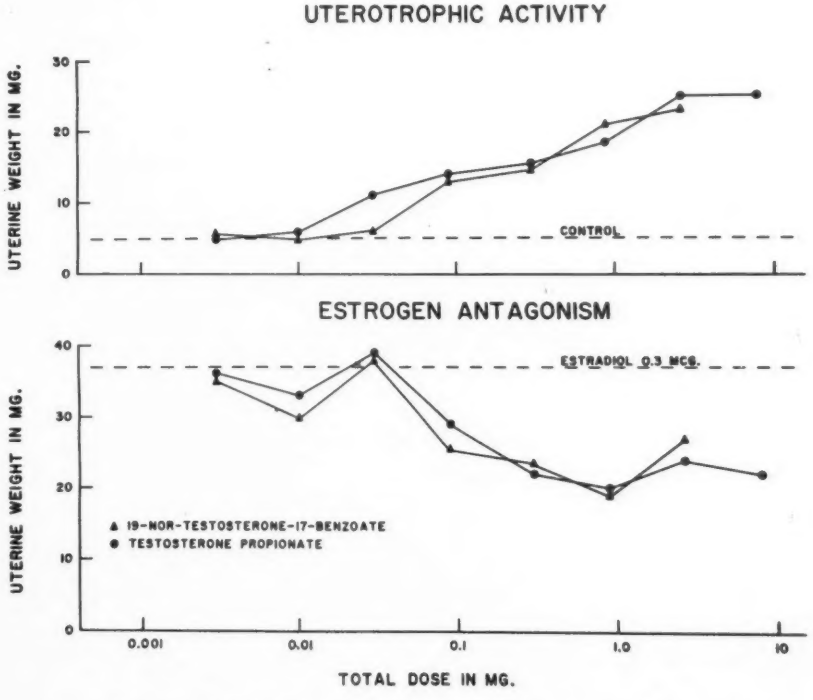

FIG. 2. Uterotrophic and anti-estrogenic activities of 19-nortestosterone-17-benzoate and testosterone propionate in immature mice.

TABLE 2. GONADOTROPHIN INHIBITION ACTIVITY OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN PARABIOTIC FEMALE RATS

Treatment	Daily dose, mg.	Castrate uterus, mg.	Intact uterus, mg.	Intact ovaries, mg.
Control		26	147	125
Testosterone Propionate	1.0	154	44	16
Testosterone Propionate	0.1	37	29	20
Testosterone Propionate	0.01	32	93	43
Testosterone Propionate	0.001	62	210	143
19-nortestosterone-17-benzoate	1.0	156	35	16
19-nortestosterone-17-benzoate	0.1	31	41	21
19-nortestosterone-17-benzoate	0.01	49	167	119

In mature male rats, 19-nortestosterone-17-benzoate was gonadotrophic inhibitory and myotrophic in doses which were not androgenic (Table 3). Testis weights of animals administered 0.5 mg./Kg. and 2.5 mg./Kg. were lower and levator ani weights were greater than those of controls. The seminal vesicles and ventral prostates weighed about the same or less than those of controls. Testosterone propionate in daily doses of 0.25 mg./Kg. and 1.25 mg./Kg. had effects on testes and levator ani similar to that of 19-nortestosterone-17-benzoate. It was more androgenic, however, since it increased the weights of ventral prostate and seminal vesicles.

Progestational activity. 19-Nortestosterone-17-benzoate and testosterone propionate had similar progestational activity (Table 4). The progestational potency of these steroids was much lower than that of progesterone.

Subchronic study of 19-nortestosterone-17-benzoate. Food consumption of intact female rats administered 19-nortestosterone-17-benzoate in doses of 3 mg./Kg. per day and 15 mg./Kg. per day and castrate female rats administered 3 mg./Kg. per day was greater than that of controls or the other experimental female group and they gained more weight. Food consumption and body weight changes for intact and castrate male rats administered the steroid were similar to that for controls. Hematologic values were similar for experimental and control groups. Organ weights (Tables

TABLE 3. EFFECTS OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN INTACT MALE RATS AFTER 10 DAILY SUBCUTANEOUS INJECTIONS

Treatment	Daily dose, mg./Kg.	Body wt. gain, gm.	Daily food intake, gm./rat	Organ weight, mg.				
				Pituitary	Seminal vesicles	Ventral prostate	Levator ani	Testes
Control (Olive oil)		46	10.1	4.1±0.3*	16.2±2.1	45.7± 5.8	30.5±3.5	1540± 82
19-nortestosterone-17-benzoate	2.5	56	11.2	4.2±0.3	15.0±0.3	27.7± 3.6	68.6±7.0	715±107
19-nortestosterone-17-benzoate	0.5	48	10.4	3.8±0.1	11.2±1.3	30.0± 1.3	40.5±1.4	880± 88
Testosterone propionate	1.25	56	11.0	4.4±0.1	136.2±9.4	151.1±12.5	73.6±2.9	721± 53
Testosterone propionate	0.25	48	10.6	5.0±0.4	32.3±2.1	57.3±10.5	41.0±1.7	982± 88

* Standard error of the mean.

5 and 6) demonstrate that 19-nortestosterone-17-benzoate prevented castration hypertrophy of the pituitary and did not significantly alter thyroid or liver weight. The adrenal weight in intact and castrate females and castrate males and the thymus weight of all rats administered 19-

TABLE 4. PROGESTATIONAL ACTIVITY OF 19-NORTESTOSTERONE-17-BENZOATE AND TESTOSTERONE PROPIONATE IN THE IMMATURE FEMALE RABBIT

Treatment*	Total dose, mg.	Uterine wt., gm.	Uterine histology†
Estrogen Primed control	—	1.14	6L
Progesterone	0.75	2.35	6H
Testosterone Propionate	27.0	2.59	6M
Testosterone Propionate	9.0	1.60	5M, 1L
Testosterone Propionate	3.0	1.25	6L
Testosterone Propionate	1.0	1.06	5L
19-nortestosterone-17-benzoate	27.0	2.42	6M
19-nortestosterone-17-benzoate	9.0	2.46	2M, 4L
19-nortestosterone-17-benzoate	3.0	1.40	6L
19-nortestosterone-17-benzoate	1.0	1.19	6L

* All animals were pretreated with 15 μg. estradiol monobenzoate.
† Number preceding letter indicates number of animals.
H = Typical progestational uterus.
M = Moderate glandular proliferation.
L = Typical estrogenized uterus; flattened glands and slight invagination.

TABLE 5. BODY AND ORGAN WEIGHTS OF MALE RATS ADMINISTERED 19-NORTESTOSTERONE-17-BENZOATE FOR 6 WEEKS

	Intact males			Castrate males		
Daily Dose, mg./Kg.	15	3	—	15	3	—
Body wt.						
Initial, gm.	139	139	128	126	119	129
Final, gm.	244	284	271	231	248	253
Pituitary, mg.	7.5± 0.4*	8.5± 0.9	7.4± 0.4	7.4± 0.5	8.6± 0.7	12.0± 0.4
Thyroid, mg.	13.0± 0.8	11.2± 1.1	11.3± 0.7	11.0± 0.7	11.4± 1.1	10.8± 1.0
Adrenals, mg.	34.3± 2.3	34.0± 2.4	32.2± 2.1	31.4± 1.2	29.8± 1.6	44.3± 2.8
Thymus, mg.	140.0±11.0	234.0±27.0	384.0±22.0	145.0±43.0	229.0±21.0	513.0±27.0
Heart, mg.	858.0±29.0	879.0±22.0	861.0±32.0	821.0±22.0	825.0±26.0	801.0±26.0
Liver, gm.	8.5± 0.3	10.6± 0.6	10.1± 0.4	7.7± 0.8	8.4± 0.3	9.5± 0.5
Kidneys, gm.	2.2± 0.1	2.1± 0.1	1.8± 0.1	2.0± 0.1	1.9± 0.1	1.7± 0.1
Testes, gm.	2.7± 0.1	1.7± 0.1	2.7± 0.1			
Seminal Vesicles, mg.	365.0±17.0	88.0± 8.0	228.0±30.0	341.0±18.0	89.0± 8.0	15.0± 1.0
Ventral Prostate, mg.	386.0±19.0	79.0±23.0	290.0±46.0	392.0±29.0	44.0± 5.0	11.0± 1.0
Levator Ani, mg.	278.0±15.0	270.0±10.0	187.0±16.0	220.0±12.0	220.0±13.0	46.0± 4.0

* Standard error of the mean.

TABLE 6. BODY AND ORGAN WEIGHTS OF FEMALE RATS ADMINISTERED 19-NORTESTOSTERONE-17-BENZOATE FOR 6 WEEKS

	Intact females			Castrate females		
Daily Dose, mg./Kg.	15	3	—	15	3	—
Body Wt.						
Initial, gm.	121	124	115	121	132	131
Final, gm.	231	234	187	229	258	231
Pituitary, mg.	8.0± 0.5*	8.2± 0.5	9.8± 0.6	9.4± 0.3	9.4± 0.4	13.5± 0.6
Thyroid, mg.	14.8± 0.8	14.8± 0.9	13.9± 0.6	15.8± 1.2	15.3± 0.5	14.9± 0.5
Adrenals, mg.	34.5± 1.5	34.5± 1.5	50.6± 4.0	35.2± 1.6	29.2± 1.3	50.4± 3.6
Thymus, mg.	89.3± 6.1	228.0±26.0	308.0±24.0	129.0±14.0	233.0±16.0	504.0±38.0
Heart, mg.	769.0± 31.0	788.0±28.0	659.0±23.0	840.0±24.0	844.0±29.0	735.0±24.0
Liver, gm.	7.4± 0.4	8.7± 0.4	6.9± 0.2	8.2± 0.4	9.0± 0.2	7.3± 0.4
Kidneys, gm.	1.8± 0.1	1.8± 0.1	1.4± 0.1	1.9± 0.1	1.9± 0.1	1.5± 0.1
Ovaries, mg.	18.7± 1.1	25.8± 2.7	56.8± 3.6			
Uterus, mg.	691.0±124.0	454.0±26	288.0±25.0	326.0±21.0	312.0±12.0	45.0± 3.0

* Standard error of the mean.

nortestosterone-17-benzoate were lower than those of the control rats. Treatment with this steroid resulted in larger kidney weights in all animals and in increased heart weights in all females. Ovarian weights were lower and uterine weights were higher than those of the control animals. Testes, seminal vesicles, and ventral prostate weights were reduced with the lower dose of the drug only.

DISCUSSION

Biological studies indicate that 19-nortestosterone-17-benzoate possesses all the activities of an androgen although end organ dose response relationships do not always follow those for testosterone propionate. The minimum dose of testosterone propionate for effecting an increase in levator ani weight in the rat also increases the seminal vesicles and ventral prostate weights. In contrast, the minimum dose of 19-nortestosterone-17-benzoate for increasing the weight of levator ani muscle does not increase accessory sex organ weights. This steroid, therefore, is similar to other 19-nortestosterone derivatives in that it produces a myotrophic effect at doses which do not increase accessory sex organ weights.

Production or release of pituitary gonadotrophins are inhibited in the male rate with doses of 19-nortestosterone-17-benzoate which do not stimulate either the ventral prostate or the seminal vesicles. Non-uterotrophic levels of this steroid almost completely suppress secretion of pituitary gonadotrophins in parabiotic female rats. These studies suggest that the pituitary is very sensitive to this compound. The anti-gonadotrophic action of 19-nortestosterone-17-benzoate can be assumed to be a property of its androgenicity since this steroid was not estrogenic in the castrate female rat.

REFERENCES

1. Eisenberg, E. and G. S. Gordan: *J. Pharmacol. and Exper. Therap.* **99**: 38. 1950.
2. Hertz, R. and R. K. Meyer: *Endocrinology* **21**: 756. 1937.
3. Greep, R. O. and I. C. Jones: *Recent Progress in Hormone Research* **5**: 197. 1950.
4. Hershberger, L. G., E. G. Shipley and R. K. Meyer: *Proc. Soc. Exper. Biol. and Med.* **83**: 175. 1953.
5. Holtkamp, D. E., A. E. Heming and L. F. Mansor: *J. Clin. Endocrinol. and Metab.* **15**: 848. 1955.
6. Barnes, L. E., R. O. Stafford, M. E. Guild, L. C. Thole and K. J. Olson: *Endocrinology* **55**: 77. 1954.
7. Saunders, F. J. and V. A. Drill: *Endocrinology* **58**: 567. 1956.
8. Byrnes, W. W. and R. K. Meyer: *Endocrinology* **48**: 133. 1951.
9. McPhail, M. K.: *J. Physiol.* **83**: 145. 1935.

ENHANCING EFFECT OF CORTICOTROPHIN ON THYROTROPHIN PRODUCED EXOPHTHALMOS IN FISH

JOSÉ M. CAÑADELL AND JOAQUIN BARRAQUER

Department of Endocrinology, Clínica Médica "B" de la Facultad de Medicina de Barcelona
and
Instituto Barraquer de Oftalmología, Barcelona (Spain)

ABSTRACT

Goldfish (*Carassius auratus*, var. *japonicus*, subvar. *bicaudatus*) respond to the administration of thyrotrophic hormone with exophthalmos. Eye protrusion increases with simultaneous injections of corticotrophin. Retrobulbar injections of normal saline, human serum or pituitary hormones produce a transient exophthalmos, which is much more persistent when thyrotrophin is injected. Previous treatment of fishes with long-acting corticotrophin prolonged the duration of the exophthalmos caused by retrobulbar injections of thyrotrophin; this effect is not observed when other fluids are injected.

SOME fish respond with an increase in the protrusion of the ocular globes as a result of different hormonal stimuli. Albert (2) produced exophthalmos in *Fundulus heteroclitus* by means of intracelomic injections of anterior pituitary extracts. Dobyns and Steelman (6) showed the possibility of obtaining the same effect by injecting preparations of pituitary glands free from thyrotrophic activity. Pickford (10), on the contrary, observed that there is a strict relation between the exophthalmos-producing and thyroid-stimulating effects of thyrotrophic preparations. Exophthalmos was also observed in common minnows in response to treatment with triiodothyronine and desiccated thyroid (8). On the other hand, Matty and co-workers (9) succeeded in producing exophthalmos by injecting androgens in two species of teleost fish (*Sparisoma squalidum* and *Scarus croicensis*) and by the injection of thyroxine and triiodothyronine in these species and in *Bathystoma aurolineatum*.

In Spain it is impossible to obtain *Fundulus heteroclitus* or any of the species of fish with which the authors mentioned above have worked. In a series of previous tests we investigated the possible exophthalmic action of anterior pituitary extracts in the following fish: *Barbus fluviatilis*, *Cyprinus carpio*, *Leuciscus erithrophthalmus*, *Fundulus hispanicus*, *Carassius auratus*, *Perca fluviatilis*, *Tinca tinca*, *Gobitis fossilis*, *Gobius fluviatilis* and *Ameiurus nebulosus*. All these, except the last, under diverse experimental conditions can develop exophthalmos following injections of thyrotrophin.

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Nevertheless we were only able to obtain *Carassius auratus* var. *japonicus* in unlimited quantities and we used this species for almost all our investigations. Between the diverse types of goldfish, the *Carassius auratus* var. *japonicus* subvar. *bicaudatus* show a greater sensitivity and uniformity of response. This subvariety has a more globular body and a flatter head. *Carassius macrophthalmus* has been derived from this fish by selection; in this species protrusion of the ocular globes, which is sometimes enormous, is an inherited characteristic (Fig. 1).

We have presented numerous clinical and experimental arguments in previous papers (4, 5) supporting the view that the exophthalmos of

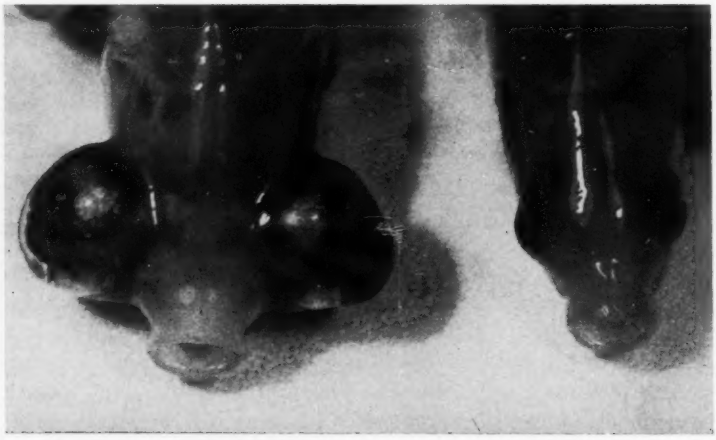

FIG. 1. Telescopic-eyed goldfish (*Carassius macrophthalmus*) with genetic constitutional exophthalmos and a common goldfish (*Carassius auratus*).

Graves' disease and other related forms of ophthalmopathy (oedematous exophthalmos, malignant exophthalmos, infiltrative ophthalmopathy, exophthalmic ophthalmoplegia, etc.) are secondary to a hyperfunction of the anterior pituitary. According to Aterman (3) the adrenal cortex also participates in the pathogenesis of these conditions. Experimenting with golden hamsters we have confirmed that the presence of adrenal glands is indispensable in the production of exophthalmos by injection of thyrotrophin and that previous treatment with corticotrophin or adrenal steroids reinforces the exophthalmic action of the thyrotrophic hormone (5).

The series of experiments that follows was planned to study the effect of corticotrophin on the exophthalmos produced in *Carassius auratus* var. *japonicus*, subvar. *bicaudatus* by intracoelomic injections of thyrotrophin and its influence on ocular protrusion following the injection of thyrotrophin directly into the retrobulbar tissues.

METHODS

Groups of ten *Carassius*, about two years old (9–10 cm. long) were injected intracoelomically at intervals of eight hours with thyrotrophic extract, the doses varying from 0.25 to 25.00 Heyl-Laqueur units. Other similar groups were injected with corticotrophin, chorionic gonadotrophin, growth hormone, human serum and normal saline solution. The treatment was continued for 5 consecutive days, during which the appearance of the fish was recorded and the protrusion of ocular globes measured with a caliper with vernier scale.

Other groups of goldfish were treated with the same doses of thyrotrophin but adding to each injection two international units of corticotrophin. Control groups were similarly treated with corticotrophin and chorionic gonadotrophin, growth hormone extract, human serum and normal saline solution.

In another experiment the exophthalmic effects of the retrobulbar injections of thyrotrophin were studied. The material was injected only once through the conjunctival sac of both eyes with an insulin syringe and a needle of small caliber. The dose was 0.05 cc. (5 U. Heyl-Laqueur). Control fish were injected with the same volume of corticotrophin, chorionic gonadotrophin, growth hormone and normal saline solution. The resulting ocular protrusion was measured immediately after the injections and at regular intervals of 8 hours.

Other fish were previously treated intracoelomically with a dose of 5 I.U. of corticotrophin-zinc following which exophthalmos was brought about in the same manner as in the preceding experiment.

RESULTS

The intracoelomic injections of thyrotrophin at doses of 10 U. Heyl-Laqueur (total quantity injected 150 U.) produced a variable exophthalmic effect. Only in two of ten injected fish was a small augmentation of the ocular protrusion and the intercorneal distance observed (1.5 mm. and 1.8 mm. respectively). Administring 25 U. by injection (total dose 375 U.), exophthalmos was observed in seven fish, in four of which it was perfectly evident on simple inspection and without the necessity of measuring intercorneal distance (Fig. 2). The increase in the intercorneal distance was 3.8 mm. ($\pm$1.15 mm.). Doses greater than 25 U. were poorly tolerated and doses of less than 10 U. had no effect. Exophthalmos was not observed in any of the fish in the control groups injected with corticotrophin, growth hormone, chorionic gonadotrophin, human serum or normal saline solution.

In the animals showing positive results, the exophthalmos began to appear on the third day of the experiment, became more evident in the two following days and disappeared completely at the end of two or three days after ceasing the injections of thyrotrophin.

We observed that the injection of corticotrophin reinforced the exophthalmic effect of thyrotrophin without increasing the intensity of the maximum result. Injections of 10 U. of thyrotrophin, with a very small exophthalmic effect (see above), produced exophthalmos in six fish with increase of the intercorneal distance of 2.9 mm. ($\pm$0.6 mm.) when it was

injected with corticotrophin. Injections of 25 U. of thyrotrophin caused exophthalmos in nine fish. The evolution of exophthalmos followed the same course as in the animals injected only with thyrotrophin without complementary treatment with corticotrophin. Control groups injected with thyrotrophin and chorionic gonadotrophin, growth hormone, human serum or normal saline solution developed exophthalmos to the same degree as the fish treated with thyrotrophic hormone alone. These materials are devoid of the enhancing effect of corticotrophin.

The retrobulbar injections of a small quantity of normal saline produced a transitory increase of ocular protrusion. An augmentation of

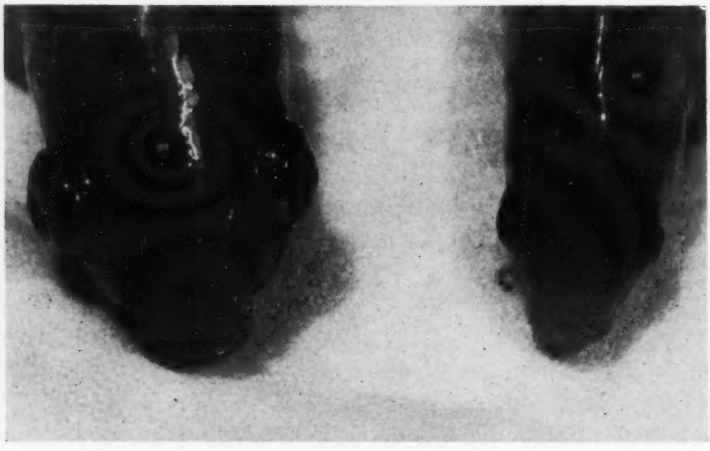

FIG. 2. Exopthalmos produced with systemic treatment with thyrotrophin. At right control fish.

5 mm. or more in the intercorneal distance could thus be obtained without ill effects. This exophthalmos persisted for some four hours, then diminished progressively and disappeared in the course of the following eight hours. Injection of gonadotrophic hormone, growth hormone or human serum produced the same phenomenon, but, possibly because of the protein composition of these fluids, the exophthalmos persisted for about 24 hours. On the contrary, the exophthalmos produced by injection of thyrotrophin was always more persistent, and after 24 hours the increase of the intercorneal distance was still 80% (±6%) of the initial value and after 48 hours it was 12% (±7%).

In the fish treated previously with corticotrophin-zinc we observed that the increase of the ocular protrusion produced by thyrotrophin was much more persistent. After 24 hours the increase in the intercorneal distance still equaled 82% (±3%) of the initial value, after 48 hours it was 58% (±5%) and after 72 hours it was 22% (±10.5%) (Fig. 3).

FIG. 3. Ocular protrusion provoked by retrobulbar injections of thryotrophin 24 hours before. The first fish on the left was previously treated with long acting corticotrophin; the middle fish did not receive corticotrophin; on the right is the control fish in which exophthalmos was produced by injecting human serum. Note the persistence of ocular protrusion in both fish treated with thyrotrophic hormone and the larger exophthalmos in the fish that received corticotrophin.

DISCUSSION

The difficulties in producing exophthalmos in some fish by injection of anterior pituitary extracts or near pure thyrotrophic preparations suggests the existence of an exophthalmic factor distinct from the thyrotrophic hormone (1, 6, 7). Nevertheless, even though it may be unjustified to apply the results of our experiments directly as a basic explanation of the pathogenesis of the exophthalmos of Graves' disease, they confirm our hypothesis (5) that this phenomenon probably develops as a result of a combination of several endocrine factors. The action of thyrotrophic hormone is the essential causative factor; the corticotrophin and the activity of the adrenal cortex have a potentiating effect, possibly by increasing the sensitivity of the retrobulbar tissues to the exophthalmogenic action of thyrotrophin. The experiments of Langford (8) and of Matty *et al.* (9) in which they showed the possibility of producing exophthalmos in fish by thyroid hormones seem to oppose the thyrotrophic hypothesis, since the administration of thyroxine produces an inhibiting effect on the pituitary secretion of thyrotrophin. However, in previous experiments (5) we observed that large doses of thyroid hormone increase the thyrotrophic exophthalmos, whilst weak doses of the same are capable of reducing it. These results—apparently paradoxical—could be explained by the toxic action of the thyroxine, which is capable of stimulating the anterior pituitary-adrenal system (11), increases the corticotrophic and corticoid secretion, and indirectly creates experimental conditions analogous to those obtained by injecting thyrotrophin and corticotrophin.

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REFERENCES

1. Aird, R. B.: *Ann. Inter. Med.* **15**: 564. 1941.
2. Albert, A.: *Endocrinology* **40**: 303. 1947.
3. Aterman, K.: *Acta Endocrinol.* supl. XX, vol. 15. 1954.
4. Cañadell, J. M. and J. Barraquer: *Rev. Inform. Méd. Terap.* **31**: 305. 1956.
5. Cañadell, J. M., and J. Barraquer: *Publ. Monog. Instituto Barraquer*, No. 4. 1957.
6. Dobyns, B. M. and S. L. Steelman: *Endocrinology* **52**: 705. 1953.
7. Friedgood, H. B.: *Bull. Johns Hopkins Hosp.* **54**: 48. 1934.
8. Langford, H. F.: *Endocrinology* **60**: 390. 1957.
9. Matty, A. J., D. Menzel, and J. E. Bardach: *J. Endocrinol.* **17**: 314. 1958.
10. Pickford, G. E.: *Endocrinology* **55**: 589. 1954.
11. Swann, H. G.: *Proc. Soc. Exper. Med. & Biol.* **40**: 520. 1939.

GROWTH-PROMOTING ACTIVITY OF PREGNANT RAT PLASMA AFTER HYPOPHYSECTOMY AND AFTER THYROIDECTOMY[1]

ALEX N. CONTOPOULOS AND MIRIAM E. SIMPSON

Institute of Experimental Biology and Department of Anatomy, University of California, Berkeley, and School of Medicine, San Francisco, California

ABSTRACT

Growth promoting potency of rat blood plasma, as evaluated by the tibial assay, decreased after thyroidectomy, and increased during normal pregnancy. Thyroidectomy did not interfere with the increased level during pregnancy. Hypophysectomy of the pregnant rat did not reduce the growth promoting activity of plasma to the extent which it did in non-pregnant rats.

Thyrotrophic activity (assayed by I^{131} uptake and thyroid morphology of hypophysectomized recipients) was demonstrable in plasma from both pregnant and non-pregnant thyroidectomized rats. Similar volumes of plasma from intact pregnant or non-pregnant rats elicited no response in the thyroids of recipients. Adrenocorticotrophic activity was not detected at the doses injected (by weight and morphology of the adrenals of recipients) in plasma from any type of donor (normal, thyroidectomized, hypophysectomized rats, pregnant or non-pregnant). Gonadotrophic activity was not detected in plasma of any type of donor, as judged by ovarian morphology of recipient hypophysectomized rats. However, judged by the follicular development in the donors, follicle-stimulating substances are present in plasma late in pregnancy both in normal-pregnant and thyroidectomized-pregnant donors, but not in hypophysectomized-pregnant donors. Luteotrophic substances were present both in hypophysectomized-pregnant and thyroidectomized-pregnant rats, as judged by maintenance of the corpora lutea in these donors.

GROWTH-PROMOTING activity has been reported in retroplacental blood plasma of women (1, 2) and more recently in blood plasma of pregnant rats (3, 4). As both hypophysectomy and thyroidectomy in the rat interfere with body growth, and as thyroidectomy has been found to decrease the growth-promoting activity both in the pituitary and in blood plasma (5, 6), any attempt to characterize the growth-promoting capacity of the plasma of pregnant animals, and trace the origin of this activity, would naturally be directed first toward the roles of the pituitary and the thyroid. It was the purpose of this study to determine the effect of hypophysectomy and of thyroidectomy on the growth-promoting potency of plasma in pregnant rats.

METHODS

Preparation of donors of plasma. Rats maintained on an adequate diet[2] were bred

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[2] All normal and pregnant rats, operated and non-operated, were maintained on a

when 90–120 days of age.[3] In order to determine the role of the thyroid, rats were thyroidectomized on day 1, 6 or 14 of pregnancy.[4] The blood plasma of normal-pregnant and of thyroidectomized-pregnant rats was bioassayed at intervals during pregnancy for growth-promoting activity. Other pituitary-like potencies of the plasma were determined simultaneoulsy. In order to determine the contribution of the pituitary to the growth-promoting activity of plasma, rats were hypophysectomized on day 12 of pregnancy and the plasma was bioassayed 4 or 8 days later. Plasma from non-pregnant normal and thyroidectomized rats of the same age and postoperative period was assayed simultaneously. The blood for bioassay was collected from the aorta and immediately centrifuged for 12 minutes, at 2000 rpm, under cover of mineral oil. The plasma was then aspirated into a syringe for injection. The time between withdrawal and injection was less than 30 minutes.

All donors were injected with 0.5 μc. of I^{131} 16–18 hours before autopsy. The thyroids from normal and hypophysectomized donors, and all tissues from the ventral neck region of thyroidectomized rats, were placed in Bouin's fixative to be counted in the scintillation counter (7). An I^{131} uptake in thyroidectomized rats greater than 0.05% of the injected dose, or 0.2% of the normal uptake, was considered evidence of incomplete removal of the thyroid, and the plasma from such animals was not used for bioassay. All pituitary target organs from donors were weighed and prepared for histological analysis of the endocrine status. Completeness of hypophysectomy was judged by examination of the pituitary site, by the weight of the pituitary target organs and by the I^{131} uptake of the thyroid. Only completely hypophysectomized rats were used as donors. Subsequently the completeness of the hypophysectomy was verified by histological analysis of the target organs.

Preparation of recipients. The animals used for assay of plasma were immature female rats of the Long-Evans strain, hypophysectomized at 26–28 days of age. They were injected when 2 weeks post-operative unless otherwise specified. Groups of these rats were injected with plasma from normal, thyroidectomized, pregnant, thyroidectomized-pregnant or hypophysectomized-pregnant rats. The doses of plasma varied from 1 to 5 cc. and were injected daily intraperitoneally for 4, 14 or 15 days. At least 4 rats were used in the assay of each dose of plasma, and each dose level was reassayed one or more times. Half of the controls were injected with comparable volumes of saline, but as no difference was observed between these and uninjected controls the values have been presented together. Body weight changes during the injection period were recorded in all recipients. Body growth was also evaluated in the rats injected for the longer periods from measurements of tail length at the beginning and the end of the experimental period. At autopsy the tibias were dissected, split and stained with silver nitrate, and the width of the uncalcified portion of the proximal epiphyseal cartilage was measured as evidence of growth stimulation (8, 9). The hypophysectomized recipients were injected

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modified McCollum Diet I, the diet used in the breeding colony, which consists of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate), 1% KI solution added to provide 1 μg. of I per gram of Diet. The hypophysectomized recipients were given daily a wet mash of the diet in addition to the dry diet. All rats received a supplement of lettuce 3 times weekly. The colony was maintained at $74 \pm 1°$ F.

[3] Female rats of the Long-Evans strain, at the proestrous stage, were caged individually overnight with males. The next morning the vaginal contents were checked and if plug and sperm were present this day was considered day 0 of pregnancy.

[4] Thyroidectomy was performed surgically, the parathyroids being removed simultaneously. Hypophysectomy was performed by the usual parapharyngeal approach.

TABLE 1. ENDOCRINE STATUS OF DONORS OF PLASMA

Group		No. of rats	Day of pregnancy		Period P.O.	Ovaries weight, mg.	Adrenals weight, mg.	Thyroid* I^{131} uptake %
			Operation	Autopsy				
Normal	Non-pregnant	35	—	—	—	64	60	14.7
	Pregnant	11	—	12	—	64	64	—
		11	—	13	—	67	56	10.0
		28	—	20	—	84	64	5.7
Hypophysectomized	Non-pregnant	9	—	—	8	31	26	0.78
	Pregnant	12	12	20	8	72	34	1.35
Thyroidectomized	Non-pregnant		—	—	6	72		0.008
		12	—	—	13	57	46	0.026
		10	—	—	14	56	45	0.015
		22	—	—	20	43	38	0.012
	Pregnant	9	6	12	6	61	61	0.005
		14	1	13	12	61	46	0.026
		11	6	20	14	89	65	0.010
		15	14	20	6	80	52	0.013
		8	1	20	19	87	47	0.025

* The values for I^{131} uptake were determined on the same day and are therefore not attributable to differences in dietary iodine intake.

with 0.5 μc. of I^{131} sixteen hours before autopsy. Their thyroids were fixed and counted in a scintillation counter, and later were sectioned and stained with hematoxylin and eosin. The thymus was dissected and weighed. The completeness of hypophysectomy of the recipients was judged by the same criteria as those used for donors.

RESULTS

The endocrine status of the donors of plasma is indicated by the data in Fig. 1 and Table 1. The normal-pregnant rats increased in body weight 98 gm. by day 20 of pregnancy (Fig. 1). The hypophysectomized-pregnant rats increased in weight by approximately the same amount by day 20 (94 gm.). Thyroidectomized-pregnant rats gained somewhat less by this

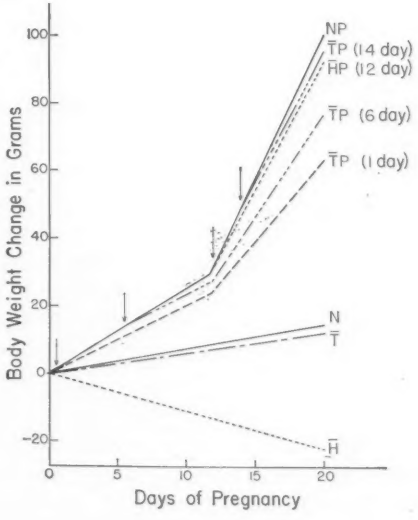

FIG. 1. Graph showing body weight gain of rats in the following categories: normal-pregnant (NP) thyroidectomized-pregnant ($\overline{T}P$) with operation performed on different days of pregnancy, as indicated by the arrows and the figures in parentheses, and hypophysectomized-pregnant ($\overline{H}P$), operation day 12 of pregnancy, as compared with appropriate non-pregnant controls: normal (N), thyroidectomized ($\overline{T}$) or hypophysectomized ($\overline{H}$); during pregnancy or a comparable 20 day period.

TABLE 2. INFLUENCE OF THYROIDECTOMY ON THE GROWTH PROMOTING ACTIVITY OF THE PLASMA OF PREGNANT RATS

Donors of plasma				Hypophysectomized recipients—14 days post-operative					
Type		Day of pregnancy	Days P.O.	No. of rats	Daily dose, 4 days	Epiphyseal cartilage width	Body weight increase	Thyroid	
								I^{131} uptake	Histology
					cc.	micra*	gm.	%	
Normal	Non-pregnant	—	—	15	3.0	201±2	2.2±0.4	0.45±.08	—
		—	—	8	3.5	202±5	2.3±0.3	0.63±.09	—
		—	—	4	4.5	211±5	2.8±0.8	0.65±.10	—
	Pregnant	12	—	5	5.1	233±4	7.0±0.7	0.56±.18	—
		13	—	8	3.5	229±4	5.9±0.4	0.60±.12	—
		20	—	8	3.0	230±4	3.0±0.5	0.52±.15	—
		20	—	4	3.7	239±3	2.0±0.6	0.72±.24	—
		20	—	3	4.4	218±3	4.0±0.8	0.66±.08	—
Thyroidectomized	Non-pregnant	—	12	4	3.0	190±3	3.0±0.4	1.87±.18	+
		—	13	8	3.0	182±2	2.6±0.5	1.90±.12	+
		—	14	4	2.3	179±4	2.2±0.5	1.46±.29	+
		—	21	5	3.4	180±3	2.6±0.4	2.88±.11	++
	Pregnant	12	6	4	3.6	224±6	6.5±1.5	1.54±.27	+
		13	13	8	3.5	233±7	4.3±0.9	1.68±.12	+
		20	6	4	4.8	220±4	3.5±0.7	1.04±.07	±
		20	8	6	2.8	221±3	1.0±0.4	0.94±.03	−
		20	13	4	4.5	235±6	3.5±0.4	1.51±.18	+
		20	14	4	2.6	213±5	5.0±1.0		
		20	14	4	3.8	237±3	2.0±0.3	0.92±.14	−
		20	20	4	2.6	221±9	2.5±1.0	1.36±.19	−
		21	21	4	3.0	244±7	5.3±1.0	1.34±.13	±
—	—	—	—	60	0	151±2	1.5±0.2	0.45±.03	−

* $SE = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

time of pregnancy (63–90 gm.) depending on the day of pregnancy when the operation was performed; those thyroidectomized on day 1 of pregnancy gained the least. Non-pregnant thyroidectomized rats of the same age gained 13 gm. in this early post-operative period, essentially the same gain as made by normal rats during the interval (15 gm.). Hypophysectomized rats lost weight (20 gm.).

The ovarian weights of the normal pregnant rats at day 13 of pregnancy were still within the range for normal rats, but by day 20 had increased from 64 mg. to 84 mg. (Table 1). This increase in ovarian weights late in pregnancy also occurred in thyroidectomized-pregnant rats, whereas in thyroidectomized rats they decreased within the same post-operative period. Hypophysectomy on day 12 of pregnancy did not result in atrophy of the ovaries by day 20 of pregnancy, whereas in non-pregnant rats ovarian atrophy followed hypophysectomy within this interval.

The adrenal weights in normal pregnant rats remained within normal range. Adrenal weights decreased after thyroidectomy in pregnant as in non-pregnant rats. After hypophysectomy the adrenals regressed to the same extent in pregnant as in non-pregnant rats.

The I^{131} uptake of the thyroids of the normal-pregnant rats on day 13 was lower than in non-pregnant rats and was reduced further by day 20. The I^{131} uptake was markedly reduced following hypophysectomy, but after hypophysectomy in pregnant rats it remained somewhat above the level in hypophysectomized controls after the same post-operative in-

TABLE 3. INFLUENCE OF HYPOPHYSECTOMY ON GROWTH PROMOTING ACTIVITY OF PLASMA AS JUDGED BY TIBIAL ASSAY

Donors of plasma				Hypophysectomized recipients—14 day post-operative			
Type	Day of pregnancy	Days P.O.	No. of rats	Daily dose 4 days	Epiphyseal cartilage width	Body weight increase	Thymus weight
				cc.	micra	gm.	mg.
	—	—	4	3.0	196 ± 3	3.3 ± 0.7	—
Normal	—	—	4	3.5	198 ± 4	3.2 ± 0.6	155 ± 6
	—	—	8	4.0	212 ± 3	4.0 ± 0.4	160 ± 5
	16	—	6	2.8	217 ± 5	3.0 ± 0.5	176 ± 6
Normal	20	—	4	3.0	220 ± 3	5.8 ± 0.6	158 ± 7
pregnant	20	—	4	3.6	245 ± 4	2.0 ± 0.3	164 ± 4
	20	—	4	5.0	226 ± 2	5.5 ± 1.0	151 ± 5
	16	4	4	3.5	225 ± 1	2.3 ± 0.4	177 ± 5
Hypophysectomized	20	8	8	3.0	210 ± 2	1.0 ± 0.1	152 ± 4
pregnant	20	8	16	3.6	230 ± 3	3.3 ± 0.4	189 ± 3
	20	8	8	4.5	225 ± 2	3.5 ± 0.5	168 ± 4
—	—	—	60	0	151 ± 2	1.5 ± 0.2	110 ± 3

terval. The I^{131} uptake was very low in thyroidectomized rats, whether pregnant or not, the low value being the basis of selection of donors.

Table 2 shows the influence of thyroidectomy on growth-promoting potency of plasma in pregnant and non-pregnant rats, as judged by the tibial assay. The potency of plasma of thyroidectomized rats was low, probably lower than in normal rats, whereas in thyroidectomized-pregnant rats it was higher than normal. The plasma from thyroidectomized-pregnant donors, though drawn at varying periods after thyroidectomy, contained the same amount of growth-promoting activity as that from normal-pregnant rats at the same period in pregnancy.

The thyrotrophic activity in plasma of thyroidectomized donors, both pregnant and non-pregnant, was increased, judged by thyroid morphology and I^{131} uptake in hypophysectomized recipients (Table 2). Daily doses of 3 to 5 cc. of plasma increased the height of the epithelial cells, caused vacuolation of colloid, and more than doubled the I^{131} uptake. Similar doses of plasma from normal rats, pregnant or non-pregnant, did not give these evidences of thyroid stimulation.

The effect of hypophysectomy on the growth-promoting activity in plasma of pregnant rats as judged by the tibia test is shown in Table 3.[5] The growth promoting potency of plasma from hypophysectomized-preg-

[5] Serum from non-pregnant hypophysectomized rats was not injected simultaneously with these experimental groups, however, the serum of rats was assayed 55 to 70 days after hypophysectomy and showed no evidence of growth promoting potency. Doses of 1, 3 and 5.5 cc. of serum were injected daily for 4 days into groups of 4 rats each. The proximal epiphyseal disc of the tibia of controls averaged 165 μ and of injected 179, 174 and 172 μ. respectively.

nant rats was greater than in normal rats, being of the order of that in normal-pregnant rats (4). The slight increases in body weights observed in hypophysectomized recipients were not considered reliable evidence for increased growth-promoting content of plasma, due to the brevity of the injection period (4 days). A more definite increase in body weight, as well as an increase in tail length, was observed when plasma obtained from rats on day 16 or 20 of pregnancy was injected for 14 or 15 days (Table 4). The plasma from both normal-pregnant and hypophysectomized-pregnant rats increased body weight and tail length to about the same extent. The width of the cartilaginous disc of the tibia was likewise increased after

TABLE 4. INFLUENCE OF HYPOPHYSECTOMY ON THE GROWTH PROMOTING ACTIVITY OF PLASMA OF PREGNANT RATS JUDGED BY BODY GROWTH

Doners of plasma				Hypophysectomized recipients					
Type	Day of pregnancy	Days P.O.	Days P.O.	Days inj.	Daily dose	Tibial cartilage width	Body weight increase	Tail length increase	Thymus weight
					cc.	micra	gm.	gm.	mg.
Normal Non-pregnant	—	—	45	14	3.0	173±6	4±1.2	2 ±0.1	123±10
Normal-Pregnant	20	—			3.0	208±6	8±1.0	5 ±0.3	152± 6
Hypophysectomized-Pregnant	20	8			3.5	221±6	6±0.8	5 ±0.3	134±10
	—	—			0	140±9	2±1.2	0.5±0.1	99± 7
Normal Non-Pregnant	—	—	30	15	3.0	175±5	11±1.0	3.5±0.2	150± 4
Normal-Pregnant	16	—			3.4	220±4	16±1.5	6.6±0.3	150± 4
Hypophysectomized-Pregnant	16	4			3.0	200±6	10±1.0	5.2±0.2	164±12
	—	—			0	143±3	0±0	0.7±0.1	99± 5

this longer injection period, and to approximately the same extent in recipients of plasma from both types of donor. The plasma from normal (non-pregnant) rats caused a smaller though consistent increase in tail length and tibial cartilage width.

The weight of the thymus was increased somewhat by injections of plasma for both 4 and 14 days, especially by plasma from pregnant rats, either normal or hypophysectomized. No gonadotrophic or adrenocorticotrophic activity was detected in the plasma from any type of donor at the doses injected, as judged by ovarian weight, follicular growth and interstitial cell repair, or by adrenal weight and cortical lipid distribution.

DISCUSSION

The persistence of greater than normal amounts of growth-promoting activity in the plasma of pregnant rats after hypophysectomy indicates a source outside the maternal pituitary. Maternal pituitary growth hormone would have disappeared from the circulation long before the period when plasma was drawn for bioassay. The anticipated period of survival of growth hormone in the circulation after hypophysectomy is relatively short as far as can be judged from injection of growth hormone, which indicated a half-life of 8–10 hours (10, 11). An increased growth-promoting

activity in plasma is here reported 4 to 8 days after hypophysectomy, that is, on days 16 to 20 of pregnancy. The fetal pituitary probably does not contribute growth hormone to the plasma in the earlier period of pregnancy (12, 13). In later pregnancy, day 20, the potency of plasma was not greater than on day 16, although it is known that the fetal pituitary by this period contains growth hormone (14). It seems probable therefore, as others have postulated previously (15), that the placenta is a source of growth-promoting substance in the hypophysectomized-pregnant rat.

After thyroidectomy of the non-pregnant rat the growth-promoting activity of plasma has been found to decrease parallel with the decrease in the pituitary growth hormone content (5, 6, 16), whereas after thyroidectomy of the pregnant rat there is no comparable decrease in growth promoting potency of the plasma. It seems probable therefore that either the origin of the growth-promoting substance or the mechanism of its release is different in the two cases. In thyroidectomized-pregnant rats the growth promoting activity present during the first half of pregnancy probably originates in the placenta, though conceivably also in the maternal pituitary. In later pregnancy the fetal pituitary may contribute.

The thyroid hormone of maternal origin does not appear to be a synergistic agent contributory to the increased growth potency of plasma of the pregnant rat. Thyroid function is actually reduced in the rat during normal pregnancy, as far as can be judged by the reduced I^{131} uptake by the thyroid. A reduction in protein bound iodine in plasma has also been reported in the rat during pregnancy (17). Furthermore, the growth-promoting activity of plasma of hypophysectomized-pregnant rats was found to be similar to that in normal pregnancy, whereas the thyroid function was reduced below that in intact-pregnant rats. The growth-promoting activity in the plasma of the thyroidectomized-pregnant rats was also similar to that in intact-pregnant rats in spite of the complete absence of thyroid tissue. Persistence in the circulation of maternal thyroxine seems unlikely, since the half-life of thyroxine is a matter of hours (18–21). At the time the plasma of thyroidectomized-pregnant rats was bioassayed and found to contain growth stimulating substances, 13 days after thyroidectomy, the amount of circulating thyroxine would therefore be insignificant. The activity of the fetal thyroid must be kept in mind in considering later pregnancy. The fetal thyroid is known to be able to accumulate iodine, and to produce and release thyroid hormone into the fetal circulation (22, 23). It is possible that fetal thyroxine may cross the placenta in amounts sufficient to synergize the growth-promoting activity of maternal plasma. Fetal thyroxine may also reinstate the growth hormone-producing acidophils of the maternal pituitary, as it is known that only small amounts of thyroxine are necessary for this effect (24, 25).

The thyrotrophic hormone content in plasma is known to increase in the non-pregnant thyroidectomized rat within the post-operative period

in question and this rise occurs only when the thyroxine level is very low (24, 25). In the present examination of the plasma of thyroidectomized-pregnant rats, the thyrotrophic hormone content was found to be increased to approximately the same level as in non-pregnant thyroidectomized rats, indicating that here too the level of thyroxine was low. Both the morphology and I^{131} uptake of the thyroids of hypophysectomized-pregnant rats indicated that thyroids of these animals were also under the influence of a thyroid stimulating substance not present in the non-pregnant hypophysectomized rat. Such thyrotrophic substances could be contributed either by the placenta or the fetal pituitary. Thyrotrophic hormone has been demonstrated to be present in the fetal rat pituitary in later pregnancy (13). The amount secreted by the fetal rat pituitary has even been shown to be capable of increasing under stimulation, as thyroid hyperplasia has been observed in fetuses of hypophysectomized-thyroidectomized pregnant rats injected with goitrogens (26). It seems less probable that the placenta produces and releases thyrotrophic substances, since it has been shown that the thyroids of decapitated fetal rats did not become hyperplastic after injection of propylthiouracil (27). This leaves the fetal pituitary as the more probable source of thyrotrophin in the hypophysectomized-pregnant rat.

Evidence for the production of some other hormones beside growth and thyroid stimulating hormones was also recorded. Enlargement of the follicles and increased ovarian weight was noted on day 20 of pregnancy in normal rats. This enlargement was probably preparatory for ovulation which is known to occur at parturition (28). The resumption of secretion of pituitary follicle stimulating hormone has been assumed to account for this follicular growth and the accompanying ovarian weight increase. The ovaries of hypophysectomized-pregnant rats did not decrease in weight as occurs after hypophysectomy in the non-pregnant rat. The ovaries even increased in size slightly later in pregnancy. The corpora lutea remained large and the luteal cells were almost as large as in intact pregnant animals, but no follicular enlargement occurred. It appears therefore that in the absence of the maternal pituitary, secretion of gonadotrophins capable of stimulating follicular growth does not occur, but that the placenta contributes a substance which maintains the corpora lutea (29, 30). In non-pregnant thyroidectomized rats the ovaries regressed and ovulation rarely occurred, but the ovaries of thyroidectomized-pregnant rats increased in weight late in pregnancy and contained large follicles as well as large corpora lutea. From this it would appear that in the thyroidectomized-pregnant rat the pituitary or the placenta, or both organs, continue to secrete the necessary gonadotrophic substances. The pituitary of the thyroidectomized rat has been shown to be able under certain circumstances to produce and release an amount of gonadotrophin comparable

to that of the normal pituitary. When, for example, gonadectomy was superimposed on thyroidectomy both the pituitary and plasma were found to contain greater amounts of gonadotrophins than after thyroidectomy alone (5, 16, 31).

REFERENCES

1. GEMZELL, C. A., F. HEIJKENSKJOLD AND L. STROM: *J. Clin. Endocrin. & Metab.* **15**: 537. 1955.
2. GEMZELL, C. A.: 3rd Symposium der Deutschen Gesellshaft fur Endokrinologie. p. 179. 1955.
3. CONTOPOULOS, A. N. AND M. E. SIMPSON: *Fed. Proc.* **15**: 39. 1956.
4. CONTOPOULOS, A. N. AND M. E. SIMPSON: *Endocrinol.* **61**: 765. 1957.
5. CONTOPOULOS, A. N., M. E. SIMPSON AND A. A. KONEFF: *Endocrinology* (In Press). 1958.
6. KNIGGE, K. M.: *Anat. Rec.* **130**: 543. 1958.
7. ANGER, H. O.: *Rev. Scient. Instr.* **22**: 912. 1951.
8. EVANS, H. M., M. E. SIMPSON, W. MARX AND E. KIBRICK: *Endocrinology* **32**: 13. 1943.
9. GREENSPAN, F. S., C. H. LI, M. E. SIMPSON AND H. M. EVANS: *Endocrinology* **45**: 455. 1949.
10. VAN DYKE, D. C., M. E. SIMPSON, C. H. LI AND H. M. EVANS: *Am. J. Phys.* **163**: 297. 1950.
11. GREENSPAN, F. S.: *J. Clin, Endocrinol.* **10**: 829. 1950.
12. JOST, A.: *Cold Spring Harbor Symposia on Quantit. Biol.* **19**: 167. 1954.
13. JOST, A. AND R. TAVERNIER: *C. R. Acad. Scien.* **243**: 1353. 1956.
14. CONTOPOULOS, A. N. AND M. E. SIMPSON: *Fed. Proc.* **16**: 103. 1957.
15. RAY, E. W., S. C. AVERILL, W. R. LYONS AND R. E. JOHNSON: *Endocrinology* **56**: 355. 1955.
16. CONTOPOULOS, A. N.: *Anat. Rec.* **130**: 288. 1958.
17. FELDMAN, J. D.: *Amer. J. Phys.* **192**: 273. 1958.
18. O'NEAL, L. W.: *Endocrinology* **53**: 358. 1953.
19. O'NEAL, L. W. AND P. HEINBECKER: *Endocrinology* **53**: 60. 1953.
20. MACLAGAN, N. F. AND J. H. WILKINSON: *J. Physiol.* **125**: 405. 1954.
21. FELDMAN, J. D.: *Endocrinology* **188**: 30. 1957.
22. GORBMAN, A. AND H. M. EVANS: *Endocrinology* **32**: 112. 1943.
23. GELOSO, J. P.: *C. R. Acad. Sci.* **246**: 168. 1958.
24. PURVES, H. D., W. E. GRIESBACH: *Brit. J. Exp. Path.* **27**: 170. 1946.
25. PURVES, H. E. AND W. E. GRIESBACH: *J. Endocrinol.* **13**: 365. 1956.
26. MANILDI, B.: Student Problem in Anatomy, Univ. of Calif. Medical School, 1958.
27. JOST, A.: *C. R. Soc. Biol.* **151**: 1295. 1957.
28. LONG, J. A. AND H. M. EVANS: The oestrous cycle in the rat and its associated phenomena. *Mem. Univ. Calif.* **6**: 34. 1922.
29. ASTWOOD, E. B. AND R. O. GREEP: *Proc. Soc. Exp. Biol. Med.* **38**: 713. 1938.
30. EVANS, H. M., M. E. SIMPSON, W. R. LYONS AND K. TURPEINEN: *Endocrinology* **28**: 933. 1941.
31. GUMBRECK, L. G.: *Anat. Rec.* **101**: 321. 1957.

GASTRIC SECRETORY ACTIVITY IN MICE AND RATS WITH TRANSPLANTED HORMONE SECRETING ADRENOCORTICAL TUMORS[1]

GERALD BLANSHARD,[2] ARTHUR I. COHEN, JULIAN ARABEHETY[3] AND SEYMOUR J. GRAY

Medical Clinics, Peter Bent Brigham Hospital, The Children's Cancer Research Foundation, The Children's Medical Center and the Departments of Medicine and Pathology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Abnormalities of gastric secretory activity have been reported in both hyper and hypo-adrenocorticoidism in man and animals. It has been suggested that different adrenocortical steroids vary in their effect upon gastric secretion.

Gastric secretory activity was studied in mice and rats with transplanted hormone secreting adrenocortical tumors, and in normal rats receiving corticosterone and desoxycorticosterone acetate. Corticosterone and 11β-OHΔ^4 androstene-3,17-dione are the principal steroids produced by the mouse adrenal tumor and corticosterone is the major steroid secreted by the rat adrenal tumor. No 17α hydroxylated adrenocortical steroids have been detected in the adrenal tumor tissues *in vivo* or *in vitro* in these animals.

There was no significant difference in gastric juice volume, pH, hydrochloric acid, potassium and chloride concentration or pepsin content in the tumor bearing animals when compared to the controls. The sodium content of the juice in tumor bearing rats was significantly elevated probably reflecting a mineralocorticoid effect. There was no difference in surface tension or rheological behavior. No pathological changes of the stomach wall were observed in either group.

Corticosterone or desoxycorticosterone acetate did not influence any parameter of gastric secretion in normal rats. These studies suggest that corticosteroids without 17α hydroxylation do not affect gastric secretory activity or induce gastric ulceration. This is in contrast to gastric secretory changes and ulceration reported in animals receiving 17α hydroxylated corticosteroids.

THE effect of adrenocortical activity upon gastric secretory function is illustrated by the abnormal gastric secretion described in clinical states of both hyper and hypo-adrenocorticoidism (1, 2, 3). Patients with

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[2] Berkeley Fellow 1957–1958 from Gonville and Caius College, Cambridge and the Middlesex Hospital Medical School, London, England.

[3] Miles-Ames Research Fellow.

the hypercorticoidism of Cushing's syndrome have been reported to secrete more gastric acid than normal after a gruel test meal (1). Patients with adrenal or pituitary over-activity (Cushing's disease) excreted abnormally high quantities of uropepsin (2, 3). Adrenocortical hyperplasia and tumors have been observed in association with multiple endocrine tumors accompanied by gastrointestinal ulceration (4).

On the other hand, adrenocortical hypofunction in man is associated with abnormally low quantities of gastric hydrochloric acid (2, 5), and peptic ulceration in Addison's disease has been observed infrequently (2, 3). Reduced gastric activity in adrenocortical insufficiency has been demonstrated in animals and man with restoration to normal levels by the administration of cortisone (2, 3, 6, 7, 8) but not by desoxycorticosterone acetate (DCA) (9). It has been suggested that cortisone-like steroids are those which affect gastric secretory activity, in contrast to corticosterone and desoxycorticosterone acetate which are without demonstrable effect (3, 10).

The development of transplantable hormone secreting adrenocortical tumors in mice and rats by Cohen *et al.* (11, 12) presented the opportunity to investigate the gastric secretory activity in these animals. Corticosterone and 11 β-OH-Δ^4 androstene-3,17-dione are the principal steroids produced by the mouse adrenal tumor, and corticosterone is the major steroid secreted by the rat adrenal tumor (12). No 17α hydroxylated adrenocortical steroids have been detected in the adrenal tumor tissues *in vivo or in vitro* (12, 13) in these animals.

MATERIALS AND METHOD

Hormone secreting adrenocortical tumors were transplanted into LAF_1 mice and into WR rats by the method previously described (11). Animals with tumor transplants and normal control animals of the same age and strain were maintained on a normal diet for six weeks in the case of the mice, and sixteen weeks in the case of the rats. At the end of this time all the mice and the majority of the rats with transplants had visible tumors. The remainder of the rats with transplants had palpable tumors.

The effect of desoxycorticosterone acetate (DCA) was evaluated by injecting intramuscularly into Sprague-Dawley female rats 2.5 mg. DCA daily for seven days. Corticosterone in doses of 2.5 mg. daily was injected intramuscularly for three days in one group and for seven days in another. A group of twenty-nine normal females were used as controls for these experiments.

Gastric juice was obtained for analysis from both mice and rats by the Shay procedure. In the rats the stomachs were removed four hours after pyloric ligation, and in the mice twelve hours after ligation. The juice was analyzed for volume, pH, hydrochloric acid, pepsin and electrolyte content as previously described (14). The significance of the difference between means was calculated using Student's "t" test and the 2% level of significance. The stomachs were examined macroscopically and microscopically.

The surface tension of gastric juice was measured using a Langmuir-Wilhelmy film balance as described by Avery *et al.* (15). Two cc. of gastric juice were diluted with 63 cc. of physiological saline for analysis. Since surface tension is a function of film area the tension was recorded at different areas, the surface being compressed to 15% of

TABLE 1. GASTRIC SECRETION IN MICE AND RATS WITH TRANSPLANTED HORMONE SECRETING ADRENOCORTICAL TUMORS

	Number of animals	Volume* (cc.)	pH	HCl		Na	K	Cl	Gastric† juice pepsin	Gastric‡ wall pepsin
				Free	Total					
				(m.eq./l.)			(m eq./l.)			
Normal mice	8	6 ±2.2	2.8 ±0.9	24 ±24	49 ±41	95 ±16	12 ± 5.5	186 ±12	21,690 ±22,000	655 ±345
Mice with tumors	12	7.1 ±1.4	2.3 ±0.6	25 ±11	56 ±30	107 ±16	10.3 ± 3.8	192 ±47	22,230 ± 6,700	671 ±780
Normal rats	7	1.2 ± .8	1.7 ±0.7	60 ±40	105 ±50	50 ± 4.2	16.6 ±11.0	156 ±11	231 ±180	1,290 ±114
Rats with tumors	14	0.85 ±0.2	2.5 ±1.3	35 ±27	84 ±25	74 ±21	12.5 ± 6.4	146 ±16	116 ± 31	1,387 ±335

Mean values with standard deviations.
* Cc. per 100 gm. body weight.
† Peptic units/cc./100 gm. body weight ×volume secreted.
‡ Peptic units/100 gm. body weight in whole stomach.

its original area. The rheological behavior was studied in a cone-and-plate type of viscometer at 37° C using shear rates of 12, 30 and 60 r.p.m. and 3 cc. centrifuged samples, pooled where necessary (16).

RESULTS

The gastric secretory activity in mice and rats with transplanted adrenocortical tumors (secreting predominantly corticosterone) is compared with control animals in Table 1. There was no statistically significant difference in volume, pH, hydrochloric acid, potassium and chloride concentration, and pepsin content of gastric juice in tumor bearing animals when compared with controls. Similarly, the pepsin content of the stomach wall was not different in the two groups. The sodium content of the juice in tumor bearing rats was significantly higher than in the control animals probably reflecting a mineralocorticoid effect. There was no change observed in volume, pH, hydrochloric acid, sodium, potassium and pepsin content of the gastric juice after the injection of corticosterone or desoxycorticosterone acetate into normal rats (Table 2).

The physical properties of gastric juice were evaluated by surface tension and viscosity measurements. Measurements of the surface tension as

TABLE 2. THE EFFECT OF CORTICOSTERONE AND DESOXYCORTICOSTERONE UPON THE GASTRIC SECRETION OF THE RAT

	Number of animals	Volume* (cc.)	pH	HCl		Na	K	Gastric† juice pepsin
				Free	Total			
				(m.eq./l.)		(m.eq./l.)		
Controls	29	3.8±1.4	1.26±0.17	93±22	127±21	33.4±21	9.3±3.6	770±340
Corticosterone (3 days)	8	2.7±0.3	1.3 ±0.1	100±10	130±20	24 ± 8	10.6±2.1	517±140
Corticosterone (10 days)	7	3.4±0.6	1.1 ±0.1	86±20	119±18	34 ±13	10.7±5	659±160
Desoxycorticosterone Acetate (7 days)	10	3.2±1.2	1.1 ±0.2	98±13	117±13	45 ±18	9.6±2.5	594±240

* Cc. per 100 gm. body weight.
† Peptic units/cc./100 gm. body weight ×volume secreted.

TABLE 3. SURFACE TENSION IN DYNES/CM. OF GASTRIC JUICE FROM RATS BEARING HORMONE SECRETING ADRENOCORTICAL TUMORS AND NORMALS

Relative area (%)	Gastric juice from rats bearing tumors			Gastric juice from normal rats	
	1	2	3	4	5
	(dynes/cm.)			(dynes/cm.)	
100	43.6	41.0	40.5	39.0	
90	36.8	36.8	35.5	34.7	41.7
80	34.8	35.0	34.2	34.3	36.8
70	34.8	34.0	34.2	32.5	34.4
60	33.0	34.0	33.0	32.0	30.7
50	33.0	33.0	32.0	29.5	28.2
40	30.5	31.8	29.5	28.2	27.0
30	29.5	30.5	27.0	24.5	25.8
20	27.0	27.5	25.0	23.2	23.3
15	27.0	25.7	24.5	22.0	22.0
20	44.0	38.6	41.5	38.0	46.6
30	49.0	42.8	44.5	42.0	
100	60.0	58.8	57.5	57.5	58.9

a function of area revealed no significant difference in the juice obtained from tumor animals and controls (Table 3). Viscosity measurements at different shear rates (Table 4) showed the rheological behavior of gastric juice of tumor animals was similar to that of controls.

Macroscopic mucosal ulceration was not seen in any of the animals in this study, and histological examination revealed no difference in the mucosa from tumor bearing animals when compared with normals.

DISCUSSION

The absence of significant changes in gastric secretory function in mice and rats with hormone secreting adrenocortical tumors (Table 1) may be attributed to the lack of 17α hydroxylated corticosteroids which have been reported to increase gastric secretory activity and acid production in animals (17, 23). The 17α hydroxylated adrenocortical steroids have not been found in the tumor bearing animals (12, 13) or in normal mice and rats in significant quantity (12, 13, 18, 19, 20, 21).

Corticosterone appears to be the predominating active steroid in both the tumor bearing animal and normal mice and rats (12, 13, 18, 19, 20, 21). The unaltered gastric secretion observed in the tumor bearing animals and

TABLE 4. THE RHEOLOGICAL BEHAVIOR OF GASTRIC JUICE FROM NORMAL MICE AND RATS AND FROM ANIMALS BEARING HORMONE SECRETING ADRENOCORTICAL TUMORS

Shear rate in r.p.m.	12	30	60
Gastric juice from tumor rat	0	5.0	10.0
Gastric juice from normal rat	1.0	6.0	9.5
Gastric juice from tumor mice	2.5	4.0	10.0
Gastric juice from normal mice	6.0	8.0	14.0

The units of measurement at different rates of shear are arbitrary and are proportional to the absolute viscosity. Note the increased deflection with increasing shear rate is similar in tumor bearing animals and controls indicating there is no significant difference in the behavior of gastric juice from the two groups.

in the normal rats receiving corticosterone and desoxycorticosterone acetate support previous findings in humans that these corticosteroids are without significant effect upon gastric secretion (2, 3). Oral corticosterone did not affect the uropepsin/creatinine ratio in humans while the 17α hydroxylated compound, hydrocortisone, increased gastric secretory activity as measured by this ratio (22). Similarly, the 17α hydroxylated adrenocortical steroids have been reported to increase gastric secretion in humans (3) and in animals (17, 23). Moreover, depressed gastric secretion in humans with adrenocortical insufficiency may be restored by cortisone administration (2, 8).

Gastric mucin, important for gastric mucosal defense, influences the physical properties of gastric juice. Both surface tension phenomena and the rheological behavior of gastric juice from tumor bearing animals and normals were the same, suggesting that under the conditions of these experiments there was no demonstrable qualitative effect on gastric mucin. Decreased gastric juice viscosity has been reported following the administration of ACTH to humans (24). Reduction in the quantity of gastric mucin has been observed histologically after cortisone and hydrocortisone (6).

The gastric mucosa in animals subjected to increased endogenous or exogenous corticosterone or exogenous desoxycorticosterone acetate in this study was free of ulceration and essentially normal both macroscopically and microscopically. This is in contrast to reports that rats given the 17α hydroxylated adrenal steroids regularly develop gastric ulceration (7, 26).

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REFERENCES

1. Kyle, J., J. S. Logan, D. W. Neill and R. B. Welbourn: *Lancet* I: 664. 1956.
2. Gray, S. J., C. G. Ramsey and G. W. Thorn: *Ann. Int. Med.* **45**: 73. 1956.
3. Gray, S. J. and C. G. Ramsey: *Recent Progr. in Hormone Res.* **13**: 571. 1957.
4. Underdahl, L. O., L. B. Woolner and B. M. Black: *J. Clin. Endocrinol. & Metab.* **13**: 20. 1953.
5. Soffer, L. J.: Diseases of the Adrenals. Second edition, p. 115, Lea & Febiger, Philadelphia, 1948.
6. Baker, B. L. and R. M. Bridgman: *Am. J. Anat.* **94**: 363. 1954.
7. Welbourn, R. B. and C. F. Code: *Gastroenterology* **23**: 356. 1953.
8. Engel, F. L.: *J. Clin. Endocrinol. & Metab.* **15**: 1300. 1955.
9. Tuerkischer, E. and E. Wertheimer: *J. Endocrinol.* **4**: 143. 1945.
10. Gray, S. J., C. G. Ramsey and R. W. Reifenstein: *New England J. Med.* **251**: 835. 1954.
11. Cohen, A. I., J. Furth and R. F. Buffett: *Am. J. Path.* **33**: 631. 1957.
12. Cohen, A. I., E. Bloch and E. Celozzi: *Proc. Soc. Exper. Biol. & Med.* **95**: 304. 1957.

13. Bloch, E. and A. I. Cohen: Personal communication.
14. Manrique, J., R. Paredes, J. Arabehety and S. J. Gray: *Am. J. Physiol.* **195**: 221. 1958.
15. Avery, M. E., J. Mead and C. A. Smith: Surface properties in the pathogenesis of atelectasis and hyaline membrane disease. In press.
16. Blanshard, G.: *Arch. Middlesex Hosp.* **5**: 222. 1955.
17. Plainos, T. C. and A. J. Philippu: *Gastroenterology* **35**: 183. 1958.
18. Done, A. K., R. S. Ely, R. B. Raile and V. C. Kelley: *Proc. Soc. Exper. Biol. & Med.* **81**: 667. 1952.
19. Hofmann, F. G. and C. Davison: *Endocrinology* **54**: 580. 1954.
20. Hofmann, F. G.: *Endocrinology* **59**: 712. 1956.
21. Southcott, C. M., H. E. Bandy, S. E. Newsom and M. Darrach: *Can. J. Biochem. Physiol.* **34**: 913. 1956.
22. Wolfson, W. Q. and G. W. Timmis: *J. Clin. Endocrinol. & Metab.* **15**: 991. 1955.
23. Woodward, E. R., L. Plzak, W. E. Trumbull and H. Schapiro: *Am. J. Dig. Dis.* **3**: 417. 1958.
24. Hirschowitz, B. I., D. H. Streeten, H. M. Pollard and H. A. Boldt: *J.A.M.A.* **158**: 27. 1955.
25. Ingle, D. J., R. Sheppard, J. S. Evans and M. H. Kuizenga: *Endocrinology* **37**: 341. 1945.
26. Ingle, D. J., M. C. Prestrud and J. E. Nezamis: *Am. J. Physiol.* **166**: 171. 1951.

THE EFFECT OF HYPOPHYSECTOMY AND OF TSH ON THE MOUSE SUBMAXILLARY IODIDE PUMP[1]

ALVIN TAUROG, G. D. POTTER AND I. L. CHAIKOFF
Department of Physiology, University of California, Berkeley, California

ABSTRACT

The I^{131} that is concentrated by the submaxillary glands of mice injected with radioiodide remains in the form of inorganic iodide, as revealed by paper chromatography and paper electrophoresis. The iodine concentrating capacity of submaxillary gland tissue in three strains of mice was determined by measuring the ratio,

$$\frac{I^{131} \text{ concentration in submaxillary}}{\text{radioiodide concentration in plasma}}$$

(S/P ratio), at various intervals after I^{131} injection. S/P ratios ranged from values of 4 to 7 for C_3H and Webster-Swiss mice to values of more than 30 for Cal A mice. In the former groups the maximum ratio was reached in about one hour, in the latter, after 4 hours.

Hypophysectomy and TSH injections did not induce any significant change in S/P ratios although, in the same mice, thyroid I^{131} uptakes or T/S ratios were markedly affected. It is concluded that the submaxillary iodide pump, unlike the thyroid iodide pump, is not directly affected by the presence or absence of TSH. These observations are discussed in relation to a previously proposed mechanism of action of TSH on the thyroid gland.

IT HAS become increasingly evident in recent years that the thyroid gland is not the only mammalian tissue which can concentrate inorganic iodide. Other tissues, such as gastric mucosa (1–6), salivary glands (2, 4, 7), lactating mammary gland (4, 8), and placenta (9, 10) have also been shown to possess mechanisms for transporting iodide against a concentration gradient. These extrathyroidal iodide pumps, like that of the thyroid, are inhibited by thiocyanate and perchlorate, a findings which led Rowlands and his coworkers (11, 12) to postulate an iodide transport mechanism for salivary tissue similar to that of the thyroid. Logothetopoulos and Myant (13) also concluded, from their radioautographic studies of the localization of iodide-I^{131} and S^{35}-labeled thiocyanate in thyroid, salivary glands and gastric mucosa, that iodide transport in these tissues operates via a common mechanism.

Since it is well known (14–16) that the thyroid iodide pump is largely under the control of pituitary thyrotropic hormone (TSH), it seemed of

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TABLE 1. SUBMAXILLARY AND THYROID I^{131} IN CAL A STRAIN MICE AT INTERVALS AFTER I^{131} INJECTION

Exp.	No. of mice per group	Age	Diet	Interval after I^{131}	Mean body wt.	Mean submaxillary wt.	Mean thyroid wt.	Submaxillary I^{131}			Thyroid I^{131}			S/P
								% of injected I^{131}	Distribution within gland		% of injected I^{131}	Distribution within gland		
									Iodide	Non-iodide		Iodide	Non-iodide	
				hr.	gm.	mg.	mg.		%	%		%	%	mean
1	2	18 mos.	Purina Lab. Chow	0.5	32.8	179	5.6	5.3	99.8	0.2	1.3	29.0	71.0	5.5
	2			2	28.5	177	5.3	11.6	99.8	0.2	4.0	10.6	89.4	14.9
	2			4	28.8	160	5.0	15.0	99.9	0.1	6.3	7.0	93.0	32.8
	2			7.25	30.2	180	4.1	5.1	99.8	0.2	—	—	—	29.5
2	4	18 mos.	Purina Lab. Chow	1	24.6	163	—	10.2	—	—	2.8	—	—	12.9
	4			2	29.6	192	—	13.8	—	—	6.5	—	—	20.1
	4			4	23.8	133	—	17.4	—	—	8.2	—	—	33.5
3	2	3 mos.	Purina Lab. Chow	2	24.4	144	—	3.3	99.9	0.1	—	—	—	8.8
	2			4	23.5	142	—	4.4	99.9	0.1	—	—	—	16.5
	2			7	22.0	118	—	1.6	99.9	0.1	—	—	—	27.8
4	4	56 days	Diet 1 (no added KI)	2	24.3	90	—	2.1	—	—	12.4	—	—	6.8
	5			4	24.2	93	—	4.9	—	—	22.6	—	—	15.9

interest to determine whether extrathyroidal iodide transporting mechanisms are subject to similar control. A convenient means of investigating this question was suggested by the demonstration (12, 17) that mouse submaxillary tissue possesses a potent iodide concentrating mechanism, both *in vivo* and *in vitro*. In the present communication, we report our findings on the effects of hypophysectomy and of TSH on the mouse submaxillary iodide pump.

METHODS

Animals—Male mice of various strains and ages were used: 1) *Cal A Strain*—Four experiments were carried out with mice of this strain. The ages and diets of the various groups are shown in Table 1. 2) *C_3H Mice*—Most of the experiments involving hypophysectomy and TSH injection were performed on mice of this strain. These animals were received at approximately 35 days of age, and placed on a diet of the following composition (Diet 1): 67.5 per cent wheat, 15 per cent casein, 10 per cent whole milk powder, 5 per cent hydrogenated vegetable oil, 1 per cent NaCl, 1.5 per cent chalk, and 50 ml. of a vitamin A- and D-containing fish oil per 8 kg. of diet. This diet is low in iodine (about 0.2 μg. I/gm.). In some experiments (Table 2) the same diet was fed with sufficient added KI to bring the level up to 1 μg. I/gm. Hypophysectomies were usually performed 1 to 2 weeks after the mice had been received, and the animals were maintained on Diet 1 throughout the experiment. 3) *Webster-Swiss Mice*—A single experiment involving two different age groups was performed (Table 3). These animals received Diet 1 with KI supplement, beginning two days before hypophysectomy.

Technique of Hypophysectomy—Hypophysectomies were performed by Mr. C. Jordan according to the technique described by Lostroh and Jordan (18). The operative mortality rate varied in different experiments. It was greatest with the young Webster-Swiss mice (50 per cent), and least with the C_3H mice (0 per cent in one experiment). Sella turcica checks were performed at autopsy with the aid of the dissecting microscope. All hypophysectomies were complete as judged by this criterion. Seven to 10 days were allowed to elapse after hypophysectomy before the mice were injected with I^{131}.

I^{131}-Injection—Oak Ridge I^{131} was purified by distillation (19). Mice received 2 to 50 μc. of distilled I^{131}, including approximately 0.3 μg. of carrier iodide. Injections were

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON UPTAKE OF I^{131} BY SUBMAXILLARIES AND THYROIDS OF C_3H MICE

Exp.	Age	Treatment	No. of mice in group	Interval after I^{131}	Body weight	Submaxillary weight	Thyroid weight	Per cent of injected I^{131}		S/P
								Submaxillary	Thyroid	
	days			hr.	gm.	mg.	mg.			
1	42	Control	5	4–5	19.3±0.56†	66± 1.3		1.5± .13	3.8 ± .43	6.5±0.12
		HypX	6	4–5	14.7±0.69	47± 4.8		3.3± .37	0.40± .024	13.8±1.7
2	57	Control	6	2	20.3±1.0	89± 3.9	1.3± .052	2.7± .35*	2.5 ± .24	4.6±0.36
		HypX	4	2	16.7±0.96	54± 4.8	1.3± .060	2.5± .32*	0.19± .017	6.2±0.90
3	55	Control	5	1	21.2±1.3	82± 5.3	1.8± .035	3.9± .52*	1.8 ± .084	7.6±0.86
		HypX	5	1	20.5±0.42	53± 2.1	1.3± .14	3.1± .31*	0.20± .017	7.9±0.83
		TSH	8	1	22.0±0.65	94± 3.3	3.1± .11	2.8± .21*	5.0 ± .33	6.2±0.57
		Control	5	4	21.6±1.3	84±10.8	1.6± .17	5.4± .94*	5.2 ± .54	11.4±1.3
		HypX	5	4	17.5±0.79	52± 3.1	1.0± .032	4.2± .70*	0.17± .016	9.4±2.0

* Paper chromatography was performed on these samples, and only a band for iodide-I^{131} was observed on the radioautograms.

† Mean ± standard error. S.E. = $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$

usually given intraperitoneally, and the mice were sacrificed from 30 minutes to 24 hours later.

Treatment of Tissues—At the conclusion of the experiment, the mice were anesthetized with Nembutal. Blood was taken directly from the heart with the chest open, and the thyroid and submaxillary glands were removed, the former with the aid of a dissecting microscope. Thyroids were rapidly weighed on a previously tared microscope slide on a semi-micro analytical balance; submaxillary glands were weighed on a torsion balance to the nearest mg.

In those experiments in which the nature of the I^{131} in the submaxillary glands was to be determined by filter paper chromatography or by paper electrophoresis, the tissue was first homogenized with bicarbonate buffer (6 to 10 vol.) containing approximately 0.001*M* thiouracil, and 20 μl. of the homogenates were applied to the filter paper strips. Chromatography was carried out in collidine-water-ammonia, and in butanol-ethanol-2N NH_4OH (20). Paper electrophoresis was performed at pH 8.3 in 0.06M veronal buffer, and at pH 6.2 in 0.05M citrate buffer.

TSH Treatment—Parke, Davis and Co. TSH (Lot 50P4) was injected into both normal and hypophysectomized mice. C_3H mice (Table 2 and Fig. (4) received subcutane-

TABLE 3. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON UPTAKE OF I^{131} BY SUBMAXILLARIES AND THYROIDS OF WEBSTER-SWISS MICE

Age	Treatment	No. of mice in group	Interval after I^{131}	Body weight	Submaxillary weight	Thyroid weight	Per cent of injected I^{131}		S/P
							Submaxillary	Thyroid	
			hr.	gm.	mg.	mg.			
55 days	Control	5	1	19.9±0.81*	78±3.7	1.8± .16	1.3 ± .24	1.0 ±0.21	4.4±0.63
	HypX	3	1	15.8±0.15	47±6.6	1.1± .017	2.1 ± .17	0.13±0.046	5.2±0.17
	TSH	6	1	19.3 0.68	85±5.3	3.4± .16	0.95± .030	8.3 ±1.1	2.7±0.26
	Control	5	4	21.1±0.29	110±2.7	1.8± .046	0.69± .18	2.2 ±0.48	3.2±0.54
	HypX	4	4	16.8±0.36	56±4.1	1.3± .17	1.5 ± .47	0.12±0.035	9.3±1.9
6 mos.	Control	5	1	31.8±1.8	196±7.1	3.3± .29	2.3 ± .27	1.5 ±0.13	3.8±0.54
	HypX	4	1	26.1±0.36	98±8.7	2.7± .25	4.3 ± .68	0.25±0.032	7.0±1.2
	Control	5	4	30.6±0.79	172±8.4	4.1± .39	1.1 ± .29	2.9 ±0.43	5.2±0.66
	HypX	5	4	27.0±1.3	89±7.3	3.1± .36	4.5 ± .93	0.15±0.035	18.0±1.7

* Mean ± standard error. S.E. = $\sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$

ous injections twice daily, each injection consisting of 0.5 mg. of TSH in 0.1 ml. isotonic saline. Webster-Swiss mice (Table 3) received injections of 1 mg. of TSH in 0.1 ml. saline twice daily. Injections were administered over a period of three days, a single TSH injection being given on the fourth day (7 injections in all). I^{131} was administered 1 to 5 hours after the last TSH injection.

Calculation of S/P Ratios—The I^{131} concentrated by mouse submaxillary tissue was present only in the form of inorganic iodide (see below). Furthermore, at early intervals after I^{131} injection, the I^{131} in plasma was also present primarily as inorganic iodide. Even in normal animals maintained on Diet 1 without added iodide, protein-bound I^{131} in plasma comprised less than 5 per cent of the total I^{131} 4 to 5 hours after I^{131} injection, as determined by precipitation with trichloroacetic acid. In the plasma of normal mice maintained on Purina laboratory chow, there was little evidence of organic I^{131} in plasma even 7 hours after I^{131} injection, as determined by filter paper chromatography. Therefore, in mice on Diet 1 sacrificed up to 4.5 hours after I^{131} injection, and in mice on Purina laboratory chow, sacrificed up to 7 hours after I^{131} injection, the ratio

$$\frac{\text{total } I^{131} \text{ per mg. of submaxillary tissue}}{\text{total } I^{131} \text{ per } \mu\text{l. of plasma}}$$

(S/P ratio) was taken as a measure of the degree to which submaxillary tissue is able to concentrate iodide.

In normal mice treated with TSH, analysis of plasma by filter paper chromatography revealed that approximately 95 per cent of the I^{131} in plasma was present as inorganic iodide 2 hours after I^{131} injection, and about 80 per cent 4 hours after I^{131} injection. In a group of C_3H mice sacrificed 24 hours after I^{131} injection (Fig. 3), 30 to 70 per cent of the plasma I^{131} was inorganic. In all of these groups, S/P ratios were based on the measured inorganic I^{131} concentration of plasma rather than on the total I^{131} concentration.

S/P Ratios in Mice Treated with Methyl Mercaptoimidazole—It seemed of interest to compare, in the same group of mice, the effect of TSH on the submaxillary iodide trap with its effect on the thyroid iodide trap. To facilitate measurement of the latter, mice were first injected with methyl mercaptoimidazole (MMI) which, in preliminary experiments, was found to block organic iodine formation in the thyroid completely at a dose of 10 mg. per 100 gm. body weight.

C_3H mice, 56 days old, that had been maintained on Diet 1 without added iodide for three weeks, were divided into four groups: normal, normal injected with TSH, hypophysectomized, and hypophysectomized injected with TSH. TSH injections were exactly as described above. Two to three hours after the last TSH injection, and nine days after hypophysectomy, each mouse received an intraperitoneal injection of MMI (10 mg. per 100 gm. body weight) and, 30 minutes later, an injection of I^{131} containing 0.3 μg. I^{127}. Animals were anesthetized with Nembutal 90 minutes after the I^{131} injection, blood was withdrawn from the heart, and thyroid and submaxillary glands were removed. S/P and T/P

$$\left(\frac{\text{thyroid } I^{131} \text{ concentration}}{\text{plasma } I^{131} \text{ concentration}}\right)$$

ratios were determined.

RESULTS

Nature of I^{131} Concentrated in Mouse Submaxillary Gland—Figure 1 illustrates paper chromatographic results obtained with submaxillary tissue from C_3H mice two hours after I^{131} injection. Results in two different chromatographing solvents are shown, and in each case, only a single

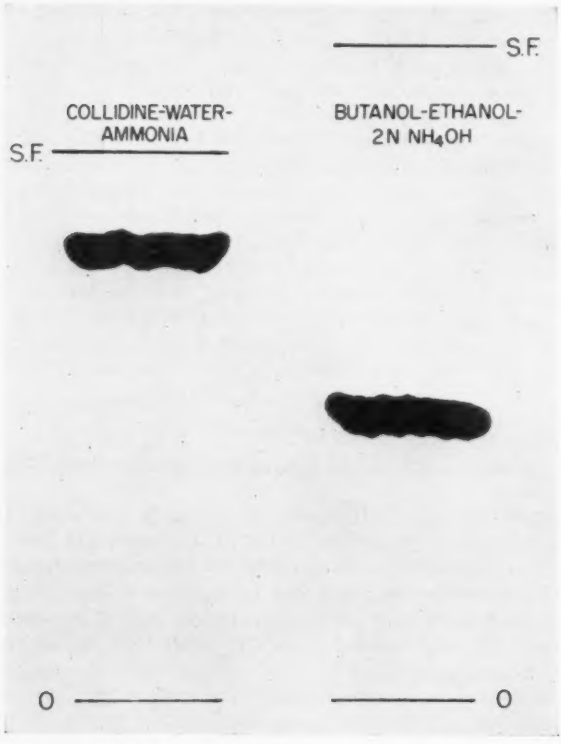

FIG. 1. Radioautographs of filter paper chromatograms of mouse submaxillary gland tissue 2 hours after injection of radioiodide. O, origin; S.F., volvent front. Inorganic I^{131} was the only component visible in the two different solvents indicated.

component was detectable on the radioautographs—that corresponding to inorganic iodide. Similar results were obtained in many other experiments, with both Cal A and C_3H mice, and at intervals from $\frac{1}{2}$ to seven hours after I^{131} injection.

Paper electrophoresis, too, was employed to examine the chemical nature of the I^{131} concentrated by mouse submaxillary gland tissue. As revealed by this procedure also, iodide-I^{131} was the only radioactive component present. Typical results are ilustrated in Figure 2, which shows the electrophoretic pattern obtained at pH 8.3 and at pH 6.2, two hours after I^{131} injection.

In no case was there any evidence of the unknown compound (U-2) which we previously encountered when cell-free preparations of rat submaxillary gland tissue were incubated with iodide-I^{131} *in vitro* (21).

Normal S/P Ratios—The highest values for S/P ratios obtained in the present study were observed in mice of the Cal A strain. As shown in Table

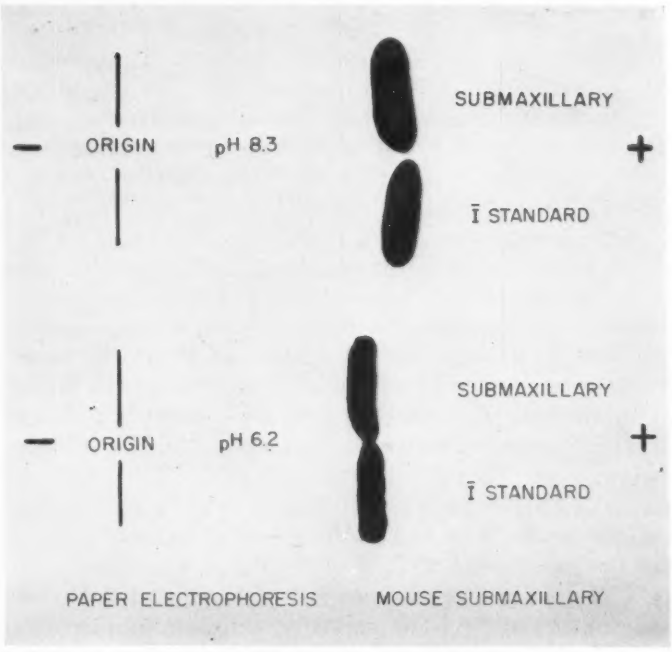

FIG. 2. Radioautographs of paper electrophoretigrams of mouse submaxillary tissue 2 hours after injection of radioiodide. Conditions of electrophoresis: Whatman No. 1 filter paper, 490 volts, 5 ma, 60 minutes duration. Buffers used: pH 8.3, 0.05M Veronal buffer; pH 6.2, 0.06M citrate buffer. Inorganic I^{131} was the only component visible at both pH's.

1, values greater than 30 were obtained in some experiments. S/P ratios in these animals appeared to increase with time, reaching a maximum value 4 to 7 hours after I^{121} injection.

With C_3H and Webster-Swiss mice, on the other hand, S/P ratios were considerably lower, and there was no consistent increase with time following the 1-hour measurement. This is clearly shown for C_3H mice in Figure 3 in which S/P ratios are plotted against time, from 1 hour to 25 hours. The mean value of 7.8 obtained for S/P at 1 hour was not significantly different from the mean value of 6.7, obtained at 24 hours. Values for S/P ratios in Webster-Swiss mice, shown in Table 3, ranged from 3.2 to 5.2, and did not change significantly between 1 and 4 hours.

S/P Ratios after Hypophysectomy—The iodide concentrating capacity of submaxillary gland tissue, as measured by the S/P ratio, was not impaired by hypophysectomy. This is shown by the data in tables 2 and 3.

Table 2 illustrates results obtained with C_3H mice in several different experiments. In no case was the S/P ratio significantly decreased after

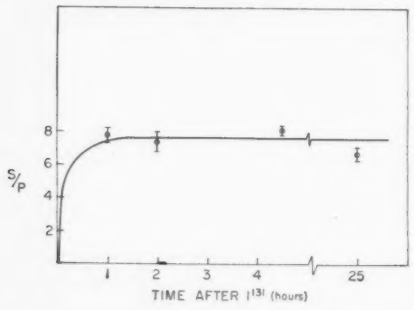

FIG. 3. Curve showing change in S/P ratio with time after I^{131} injection in normal C_3H mice. Each point represents the mean of results obtained with 4 or 5 animals. Vertical lines passing through points indicate standard error of the mean.

hypophysectomy, although thyroid uptakes of I^{131} in the same animals were greatly reduced. Results of similar experiments with Webster-Swiss mice are shown in Table 3. In the latter, an increase in S/P was usually observed after hypophysectomy. Possible reasons for this increase are discussed below.

S/P Ratios in TSH-Injected Mice—Injections of TSH into either normal or hypophysectomized mice had no significant effect on S/P ratios. This is illustrated by Experiment 3, Table 2, in which administration of TSH to normal C_3H mice had no effect on S/P ratios, although thyroid weight and thyroid uptake of I^{131} were markedly increased. Similar observations are recorded for Webster-Swiss mice in Table 3.

In Figure 4, effects of TSH injection on both S/P ratios and thyroid I^{131} uptakes are compared in normal and in hypophysectomized C_3H mice at various intervals after I^{131} injection. The effect of TSH administration on thyroid I^{131} uptake is immediately apparent, but the salivary iodide concentrating mechanism in the same animals was not affected by treatment with the trophic hormone.

S/P and T/P Ratios in Mice Treated With Mercaptoimidazole—Thyroid and submaxillary iodide traps were compared in four groups of mice, as described above under "Methods." Organic binding of I^{131} was blocked in these animals by methylmercaptoimidazole (MMI). The results, summarized in Table 4, show that, under these conditions also, hypophysectomy had no effect on S/P ratios. T/P ratios, however, were reduced by hypophysectomy to approximately $\frac{1}{5}$ the control value, a result in agree-

TABLE 4. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON S/P AND T/P RATIOS IN C_3H MICE TREATED ACUTELY WITH METHYLMERCAPTOIMIDAZOLE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight	S/P	T/P
Normal controls	7	22.1 ± .73	70 ± 4.8	1.41 ± .050	20.5 ± 2.7	350 ± 28
HypX	7	18.4 ± .84	39 ± 2.1	1.16 ± .058	19.6 ± 3.6	76 ± 12
Normal + TSH	6	22.1 ± .66	78 ± 6.3	2.84 ± .056	14.0 ± 1.1	142 ± 10
HypX + TSH	7	15.7 ± .99	37 ± 5.0	1.70 ± .11	20.6 ± 1.7	156 ± 17

ment with observations previously made by Wollman and Scow (16). Administration of TSH to hypophysectomized mice partially restored T/P ratios, but had no significant effect on S/P ratios. The interesting finding that administration of TSH to normal mice significantly reduced T/P ratios agrees with the previous report of Scow and Greer (22) who observed a similar reduction in mice fed propylthiouracil (PTU). A possible explanation was provided by Halmi (6) who postulated that if "iodide acceptor sites" in the thyroid are already near a maximum, they may not be increased by excess TSH, whereas this hormone might, at the same time, still exert its usual effect on the weight of the gland.

A surprising result encountered in the experiment of Table 4 was the

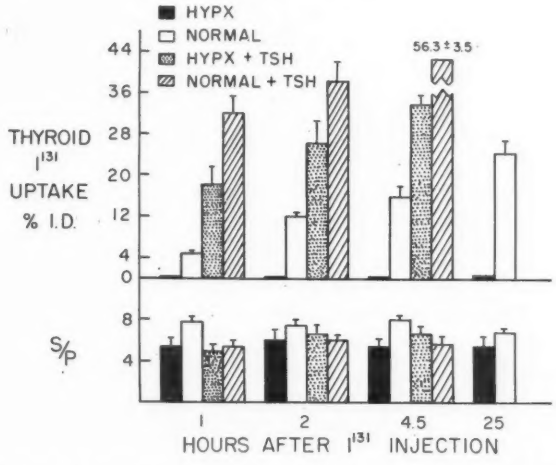

FIG. 4. Bar graph showing comparative effect of TSH administration on S/P ratios (lower section) and thyroid I^{131} uptakes (upper section) in normal and hypophysectomized C_3H mice. Each bar represents the mean of measurements made on 4 or 5 animals. The vertical line above each bar indicates the positive portion of the standard error of the mean.

unexpectedly high value for S/P. The mean value was 20.5, more than twice the value obtained for similar mice not treated with MMI (Fig. 3). This raised the question whether MMI had some specific effect on the submaxillary iodide trap, and a separate experiment was performed to compare the effect of MMI with that of PTU on S/P and T/P ratios. Normal C_3H mice of the same age and maintained on the same diet as those employed above were divided into four groups: controls, saline-injected, MMI-injected and PTU-injected. MMI and PTU were given in approximately equimolar dosage, the former at 10 mg. per 100 gm. body weight, the latter at 15 mg. per 100 gm. body weight. I^{131} containing 0.3 μg. of stable iodide was injected 30 minutes after the saline or antithyroid drug,

TABLE 5. COMPARISON OF EFFECT OF METHYLMERCAPTOIMIDAZOLE AND PROPYLTHIOURACIL ON S/P AND T/P RATIOS IN NORMAL C_3H MICE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight	S/P	T/P
None	6	21.3 ± .58	62 ± 2.6	1.27 ± .10	6.2 ± .52	
Saline	6	21.3 ± .81	58 ± 4.0	1.28 ± .062	6.6 ± .77	
MMI	6	21.7 ± .71	61 ± 1.8	1.31 ± .11	13.2 ± .60	345 ± 49
PTU	6	21.7 ± .71	63 ± 2.6	1.48 ± .13	6.3 ± .42	283 ± 35

and the animals were sacrificed one hour later. As shown in Table 5, S/P ratios in MMI-treated mice were again significantly greater than in control mice, whereas injection of PTU, or of saline alone, had no effect. T/P ratios in the MMI-treated mice, on the other hand, were not significantly different from those of PTU-treated mice. This observation suggests that MMI has some specific effect on mouse submaxillary gland tissue not shared by PTU. Further experiments are necessary to establish the mechanism of this effect.

Effect of Hypophysectomy on Submaxillary and Thyroid Weights—In mice deprived of their pituitaries, body, thyroid, and submaxillary weights were significantly reduced below control values (tables 2 and 3). Relative weights (i.e., weights per 100 gm. body weight) of thyroid and submaxillary were calculated for C_3H mice in several of the groups studied here, and the combined results are summarized in Table 6. Although absolute thyroid weights declined significantly after hypophysectomy, it is evident from Table 6 that there was no significant change in the relative weight of the thyroid. However, it was found that hypophysectomy resulted in a significant reduction in the relative weight of the submaxillary gland. This reduction was not reversed by administration of TSH either to normal or to hypophysectomized mice. Thyroid weight, on the other hand, did increase significantly in both groups treated with TSH.

Loss in relative weight of submaxillary tissue after hypophysectomy was previously noted in the mouse by Lacassagne and Chamorro (23), and in the rat by Gabe (24) and by Baker *et al.* (25, 26). In both species the principal site of regression was the serous tubules. Hypophyseal control

TABLE 6. EFFECT OF HYPOPHYSECTOMY AND OF TSH ON THYROID AND SUBMAXILLARY WEIGHTS OF C_3H MICE

Treatment	Number of mice in group	Body weight	Submaxillary weight	Thyroid weight
			mg. per 100 gm. body wt.	mg. per 100 gm. body wt.
Normal controls	26	19.7 ± .56	326 ± 28	6.3 ± .17
HypX	27	15.7 ± .51	233 ± 22	6.5 ± .16
Normal + TSH	19	20.0 ± .56	352 ± 37	12.9 ± .43
HypX + TSH	19	15.0 ± .56	228 ± 32	10.6 ± .52

over rat salivary tubular cells probably occurs by way of the thyroid and the testes (24–27). In hypophysectomized mice, it has been reported that testosterone alone restores the submaxillary tubular system (23). The role of thyroid hormone in the maintenance of mouse submaxillary serous tubules remains to be determined, especially since we observed no restoration of submaxillary weight in hypophysectomized mice treated with TSH.

DISCUSSION

The nature of the I^{131} secreted in the saliva of human subjects was investigated by Honour, Myant, and Rowlands (4), who found that although an appreciable part of the I^{131} was sometimes precipitable with trichloroacetic acid, the same was true of I^{131}-iodide added *in vitro* to saliva. The inference was that all the I^{131} in the saliva was iodide. Freinkel and Ingbar (7) chromatographed saliva obtained from patients within three hours after I^{131} injection, and concluded that all of the I^{131} was present in inorganic iodide. Fletcher, Honour, and Rowlands (12) chromatographed butanol extracts of mouse submaxillary tissue, labeled with I^{131} both *in vivo* and *in vitro*, and observed only iodide-I^{131} on their chromatograms. However, I^{131}-protein, had it been present, would have escaped detection by their butanol extraction procedure. Logothetopoulos and Myant (13) reported that chromatograms of whole homogenates of hamster salivary glands, removed after I^{131} injection, showed a large peak for iodide (about 90 per cent), and a small peak at the origin. They did not comment on the possible significance of the radioactivity at the origin. Gerbaulet and Fitting (28) used paper electrophoresis to analyze I^{131} in the saliva of a hyperthyroid patient. Although they observed that 10 to 15 per cent of the I^{131} was not inorganic, even as early as 30 minutes after I^{131} injection, they were unwilling to conclude that the noninorganic I^{131} had any physiological significance. Ruegamer (29) chromatographed the saliva of dogs after injection of I^{131}-labeled iodide, diiodotyrosine, thyroxine, or triiodothyronine. In all instances he found only I^{131}-iodide appearing in the saliva.

Our own findings (Figs. 1, 2) lead us to conclude that, at least during the early intervals after I^{131} injection, practically all of the I^{131} which appears in the submaxillary gland tissue of mice is inorganic. Only a few animals were observed at intervals as long as 24 hours, and, in these, there was a significant component (1.4 to 6.3 per cent of the total I^{131}) at the origin of the paper chromatogram, suggesting the presence of a small amount of I^{131}-protein. Further experiments are necessary to establish the significance of this fraction. Saliva from only a single human subject has been chromatographed in our laboratory—a patient suspected of hyperthyroidism, but who turned out to have a low I^{131} conversion ratio. Twenty-four hours after I^{131} administration, the I^{131} in saliva was entirely inor-

ganic. At the 96-hour interval, when the I^{131} in plasma was 52 per cent protein-bound, the saliva of this patient showed only a trace of I^{131} at the origin of the chromatogram, the remainder being present as inorganic iodide. Considering all the evidence at hand, we believe that there has been no convincing demonstration that organic I^{131} is present in the saliva or in salivary gland tissue of animals injected with I^{131}. However, rat submaxillary gland homogenates and subcellular fractions, incubated *in vitro* with I^{131}, do seem to be capable of forming organic I^{131} (12).

Interest in salivary secretion of I^{131} was stimulated by the report of Thode *et al.* (30) that there is excellent correlation between clinical thyroid status and the ratios obtained when I^{131} concentration in mixed saliva is related either to total or to protein-bound I^{131} in plasma 24 hours after I^{131} administration. These workers suggested that at least part of the I^{121} which appears in saliva of humans is derived from hormonal iodine of plasma through the action of an enzymatic iodinating-deiodinating system present in salivary gland tissue (31). The latter suggestion has not been confirmed by other investigators (29, 32–34). Moreover, observations on patients in many different laboratories (32, 35–38) indicate that there is no consistent correlation between thyroid function and salivary iodide concentrating ability. Gerbaulet and Fitting (28, 39, 40), although they reported that saliva/plasma I^{131} concentration gradients are significantly higher in hyperthyroids than in euthyroids, did not attribute this to any direct effect of increased thyroid function on salivary gland tissue, but rather to a depressed level of circulating inorganic iodide. Wase and Feng, in a brief report (41), stated that salivary glands in the mouse may play an important part in the regulation of thyroid activity, presumably through "control of production and/or utilization of thyroid stimulating hormone." This work requires confirmation.

In the present study, we used the ratio

$$\frac{I^{131} \text{ per mg. submaxillary tissue}}{\text{Iodide-}I^{131} \text{ per } \mu\text{l. plasma}}$$

as an index of the iodide concentrating capacity of salivary tissue, and compared this ratio in normal, hypophysectomized, and TSH-treated mice. Hypophysectomy did not alter the S/P ratio in any consistent manner, although in experiments with Webster-Swiss mice the ratio increased in most cases after removal of the pituitary. Since S/P ratios in humans (42) and in dogs (43) have been shown to vary inversely with salivary flow rate, it is possible that the high S/P values observed in the hypophysectomized Webster-Swiss mice may be related to a reduced salivary flow rate following pituitary removal. If this explanation is valid, it is not clear why similar increases in S/P were not also observed in C_3H mice, unless, of course, a simultaneous decrease in iodide concentrating

capacity occurred in these animals. If such a decrease occurred, it was not reversible by administration of TSH and, therefore, could not be attributed to TSH lack.

The use of whole submaxillary gland tissue for the calculation of S/P ratios, rather than of saliva itself, as employed by most previous workers, raises the question of the storage of saliva within the tissue and how this might have been affected by the procedures used here. Logothetopoulos and Myant (13) showed that, in hamsters and mice, the mixed saliva /plasma I^{131} concentration ratio was much higher than the simultaneously determined submaxillary/plasma I^{131} concentration gradient. It is apparent, therefore, that the magnitude of the S/P ratio, as calculated in the present study, depends a great deal on the amount of saliva present in the lumen of the ducts at the time the tissue is removed. Perhaps the high S/P ratios observed in the Cal A strain mice (Table 1) are related to a large pool of saliva contained within the duct system at any given time. This could also explain the slower approach to maximum S/P observed in this group, since a longer time would be required to label a larger pool of saliva. The effect of hypophysectomy on storage of saliva within the submaxillary ducts was not determined in the present study, and we cannot, therefore, assess the contribution of this factor to the S/P values obtained after hypophysectomy.

In view of the above considerations, a simple interpretation of S/P ratios as measured here does not seem warranted. However, it seems reasonable to conclude from our findings that pituitary function does not exert any direct control over salivary iodide transport comparable to that exercised over the thyroid iodide "trap." Furthermore, if there be any pituitary control over salivary iodide secretion, it is not effected through TSH. Administration of TSH, although it produced very striking increases in thyroid function (Fig. 4), did not alter S/P values. Fletcher *et al.* (12) previously observed that TSH, added *in vitro*, had no effect on I^{131} uptake by surviving slices of mouse submaxillary glands. Halmi (6, 44) and Brown (45) have reported that the gastric iodide pump of the rat also does not respond to TSH injections. The available evidence indicates, therefore, that extrathyroidal iodide pumps are not affected by TSH in the same manner as the thyroid iodide pump.

Previous studies in our laboratory (46, 47) led us to conclude that the primary action of TSH on the thyroid is not on iodine metabolism *per se*, but rather on some fundamental thyroid cellular process which, in turn, affects the various phases of iodine metabolism independently. From this point of view TSH would not be expected to affect the iodide pumps of extrathyroidal tissues, particularly since histological studies by Honour *et al.* (4) and by Logothetopoulos and Myant (13) did not reveal any cell types which are common to the various tissues that display iodide trans-

porting mechanisms. The uniqueness of TSH probably lies in its ability to stimulate the activity of thyroid cells, and it does not directly stimulate the cells of those tissues which may share with the thyroid a common mechanism for transporting iodide against a concentration gradient.

Thyrotropin has been reported to possess extrathyroidal actions such as exophthalmic (see Sonenberg (48) for discussion) and adipokinetic (49) activity. Although the exophthalmic activity has been reported to be separable from TSH activity (50), the adipokinetic activity is still retained in highly purified TSH preparations (51). The biological significance of these extrathyroidal actions of TSH remains to be determined.

REFERENCES

1. Davenport, H. W.: *Gastroenterology* **1**: 1055. 1943.
2. Schiff, L., C. D. Stevens, W. E. Molle, H. Steinberg, C. W. Kumpe and P. Stewart: *J. Natl. Can. Inst.* **7**: 349. 1947.
3. Mason, E. E. and H. S. Block: *Proc. Soc. Exper. Biol. and Med.* **73**: 488. 1950.
4. Honour, A. J., N. B. Myant and E. N. Rowlands: *Clin. Sci.* **11**: 447. 1952.
5. Logothetopoulos, J. H. and N. B. Myant: *J. Physiol.* **133**: 213. 1956.
6. Halmi, N. S.: *Ciba Foundation Colloquia on Endocrinology* **10**: 79. 1956., J. & A. Churchill Ltd., London.
7. Freinkel, N. and S. H. Ingbar: *J. Clin. Invest.* **32**: 1077. 1953.
8. Brown-Grant, K.: *J. Physiol.* **131**: 70. 1956.
9. Logothetopoulos, J. and R. F. Scott: *J. Physiol.* **132**: 365. 1956.
10. Hall, P. F. and N. B. Myant: *J. Physiol.* **133**: 181. 1956.
11. Edwards, D. A. W., K. Fletcher and E. N. Rowlands: *Lancet* **266**: 498. 1954.
12. Fletcher, K., A. J. Honour and E. N. Rowlands: *Biochem. J.* **63**: 194. 1956.
13. Logothetopoulos, J. H. and N. B. Myant: *J. Physiol.* **134**: 189. 1956.
14. VanderLaan, W. P. and M. A. Greer: *Endocrinology* **47**: 36. 1950.
15. Halmi, N. S., B. N. Spirtos, E. M. Bogdanove and H. J. Lipner: *Endocrinology* **52**: 19. 1953.
16. Wollman, S. H. and R. O. Scow: *Endocrinology* **53**: 332. 1953.
17. Collins, K. J. and K. Hellmann: *J. Physiol.* **128**: 49P. 1955.
18. Lostroh, A. J. and C. W. Jordan, Jr.: *Proc. Soc. Exper. Biol. and Med.* **90**: 260. 1955.
19. Taurog, A., G. D. Potter and I. L. Chaikoff: *J. Biol. Chem.* **213**: 119. 1955.
20. Taurog, A. and I. L. Chaikoff: In "Methods in Enzymology" Vol. IV, ed. S. P. Colowick and N. O Kaplan, Academic Press Inc , New York, p. 856. 1957.
21. Taurog, A., W. Tong and I. L. Chaikoff: *J. Biol. Chem.* **227**: 759. 1957.
22. Scow, R. O. and M. A. Greer: *Endocrinology* **56**: 590. 1955.
23. Lacassagne, A. and A. Chamorro: *Compt. rend. Soc. Biol.* **134**: 223. 1940.
24. Gabe, M.: *Arch. d'Anat. microscop. et Morphol. exptl.* **39**: 15. 1950.
25. Baker, B. L. and G. D. Abrams: In "The Hypophyseal Growth Hormone: Nature and Actions" McGraw-Hill Book Co., New York, p. 107. 1955.
26. Baker, B. L. and E. C. Pliske: In "Symposia Soc. Exp. Biol." Vol. XI, Cambridge University Press, London, p. 329. 1950.
27. Grad, B. and C. P. Leblond: *Endocrinology* **45**: 250. 1949.
28. Gerbaulet, K. and W. Fitting: *Klin. Woch.* **34**: 120. 1956.
29. Ruegamer, W. R.: *Proc. Soc. Exper. Biol. and Med.* **90**: 146. 1955.
30. Thode, H. G., C. H. Jaimet and S. Kirkwood: *New Eng. J. Med.* **251**: 129. 1954.

31. Fawcett, D. M. and S. Kirkwood: *J. Biol. Chem.* **209:** 249. 1954.
32. Freinkel, N. and S. H. Ingbar: *New Eng. J. Med.* **252:** 125. 1955.
33. Myant, N. B.: *J. Physiol.* **133:** 603. 1956.
34. Tong, W., G. D. Potter and I. L. Chaikoff: *Endocrinology* **57:** 636. 1955.
35. Fellinger, K., R. Höfer and H. Vetter: *J. Clin. Endocrinol. and Metab.* **16:** 449. 1956.
36. Gabrielsen, Z. and A. L. Kretchmar: *J. Clin. Endocrinol. and Metab.* **16:** 1347. 1956.
37. Ferguson, M. H., A. Naimark and J. A. Hildes: *Can. Med. Assoc. J.* **77:** 578. 1957.
38. Stein, J. A., Y. Feige and A. Hochman: *J. Lab. Clin. Med.* **49:** 842. 1957.
39. Gerbaulet, K. and W. Fitting: *Klin. Woch.* **35:** 576. 1957.
40. Gerbaulet, K. and W. Fitting: In "Radioaktive Isotope in Klinik und Forschung" Ed. K. Fellinger and H. Vetter, Supplement to Strahlentherapie **36:** p. 169. 1957.
41. Wase, A. W. and Y. S. L. Feng: *Nature* **177:** 624. 1956.
42. Ferguson, M. H., A. Naimark and J. A. Hildes: *Can. J. Biochem. and Physiol.* **34:** 683. 1956.
43. Burgen, A. S. V. and P. Seeman: *Can. J. Biochem. and Physiol.* **35:** 482. 1957.
44. Halmi, N. S. and R. G. Stuelke: *Endocrinology* **64:** 103. 1959.
45. Brown, J.: *Endocrinology* **58:** 68. 1956.
46. Taurog, A., W. Tong and I. L. Chaikoff: *Endocrinology* **62:** 664. 1958.
47. Taurog, A., W. Tong and I. L. Chaikoff: *Ciba Foundation Colloquia on Endocrinology* **10:** 59. 1956., J. & A. Churchill Ltd., London.
48. Sonenberg, M.: in *Vitamins and Hormones* **16:** 205. 1958. Academic Press Inc., New York.
49. Engel, F. L., M. G. Engel and H. T. McPherson: *Endocrinology* **61:** 713. 1957.
50. Dobyns, B. M. and S. L. Steelman: *Endocrinology* **52:** 705. 1953.
51. Steelman, S. L.: personal communication.

FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The First International Congress of Endocrinology will be held in Copenhagen, Denmark, July 18–23, 1960.

United States members of the International Executive Committee are Dr. Warren O. Nelson and Dr. Leo T. Samuels. Dr. Gregory Pincus is Chairman of the Program Committee. The tentative program consists of ten symposia covering the general field of endocrinology plus round table discussions and a number of submitted papers. Those desiring to submit papers should immediately contact Dr. Gregory Pincus, Worcester Foundation, Shrewsbury, Massachusetts. There will be a number of interesting scientific and technical exhibits.

In order to facilitate travel arrangements to the Copenhagen Convention, the Council of The Endocrine Society at its San Francisco meeting designated THOMAS COOK & SON as our authorized travel agents. They will in turn offer especially designed All Expense Paid Tours to Europe, including hotel reservations for the convention, as well as offering independent itineraries for any of our members who desire to travel separately by air or by steamship.

Registration is open to anyone interested in endocrinology at an approximate fee of $28 U. S. Members of the families of those attending may register as associates at an approximate fee of $14 U. S., fee to be paid at the time of registration or in advance to the American Express Company, which has been designated by the Executive Committee of the International Congress as official financial and travel agent. There will be a number of social functions and other entertainment provided for all registrants.

As you all know, European travel is unusually heavy during July and it is suggested that those contemplating attending the Congress should start making tentative reservations at the earliest possible moment by filling in and returning the card recently mailed to members, to THOMAS COOK & SON's special representative—Harry E. Kornbaum, Transportation Chairman, Rainbow Travel Service, 2817 Classen Boulevard, Oklahoma City 6, Oklahoma, who will correspond directly with each member about his individual travel arrangements to and from the Convention, at the same time keeping our National Officers fully informed of all travel arrangements of its members. Completion of the card CARRIES NO OBLIGATION OR COMMITMENT ON YOUR PART. Send it in today for the complete travel arrangements for our Society.

The general expenses of a meeting of this magnitude are quite large and

The Endocrine Society has made a substantial contribution from its treasury for organizational purposes. U. S. pharmaceutical firms have also contributed very generously; however sufficient funds are not as yet available to assure the success of this meeting; therefore, the committee suggests that each of our members make voluntary contributions to this fund. Please make your checks payable to: The Endocrine Society—First International Congress of Endocrinology. Mark it *Contribution* and mail it to the office of the Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

NO. 6. IMPLANTATION OF OVA

The attention of members of the Endocrine Society is drawn to the publication of this Memoir which reports the proceedings of a Symposium held in London to survey nidation of the mammalian ovum. The various factors—hormonal, biochemical and pharmacological—concerned are discussed in the light of recent research, most of it previously unpublished.

The volume consists of about 100 pages of text and is extensively illustrated with text-figures and plates.

Copies may be obtained at a price of $6.00 through booksellers or from the publishers, Cambridge University Press, 32 East 57th Street, New York 22, N. Y.

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Inter-Society Cytology Council, Annual Scientific Meeting, Statler Hilton Hotel, Detroit, Michigan, November 19, 20, 21, 1959, Paul A. Younge, M.D., Secretary, 1101 Beacon Street, Brookline 46, Mass.

AUTHOR INDEX, VOLUME 64

ALBERT, A., see FREY, HARALD M., 304

ALBERT, H., see HALMI, N. S., 618

ALBRIGHT, EDWIN C., KENKICHI TOMITA AND FRANK C. LARSON. *In Vitro* Metabolism of Triiodothyronine, 208

ANDERSON, CHARLES H., MICHAEL MCCALLY AND GORDON L. FARRELL. The Effects of Atrial Stretch on Aldosterone Secretion, 202

APPASWAMY, M. RAO, see MEYER, ROLAND K., 890

ARABEHETY, JULIAN, see BLANSHARD, GERALD, 1032

ARGYRIS, BERTIE F. The Mechanism of Insulin Resistance in the KL Strain of Mice: Inactivation of Insulin, 400

ASKONAS, B. A., see INGBAR, S. H., 110

ASLING, C. WILLET, PATRICIA W. DURBIN, MURIEL E. JOHNSTON AND MARSHALL W. PARROTT. Demonstration of the Concentration of Astatine-211 in the Mammary Tissue of the Rat, 579

ASPINALL, RICHARD L., see MEYER, ROLAND K., 890

AURBACH, G. D. The Influence of Gelatin in the Assay of Purified Parathyroid Extracts, 296

BACK, NATHAN, see SANDBERG, AVERY A., 775

BAGGET, BILLY, LEWIS L. ENGEL, LUZMARIA BALDERAS AND GERTRUDE LANMAN. Conversion of C^{14}-Testosterone to C^{14}-Estrogenic Steroids by Endocrine Tissues, 600

BAJUSZ, EÖRS. Effect of Hormones upon Regression of Muscle Atrophy of Nervous Origin, 262

BALDERAS, LUZMARIA, see BAGGETT, BILLY, 600

BARKER, S. B., see PITTMAN, C. S., 466

BARKER, S. B., see ETLING, NICOLE, 753

BARRAQUER, JOAQUIN, see CAÑADELL, JOSÉ, M., 1017

BATTS, ADRIENNE A., STEPHEN A. GAAL AND DALE L. TIPTON, JR. Changes in the Golgi Apparatus of the Islets of Langerhans in the Rat Following Glucose and Insulin Administration, 503

BATTS, ADRIENNE A. Effect of Hypophysectomy and Growth Hormone Administration upon the Golgi Apparatus in the Islets of Langerhans in the Rat, 610

BECKER, DAVID V. AND JOHN F. PRUDDEN. The Metabolism of I^{131}-Labeled Thyroxine, Triiodothyronine and Diiodotyrosine by an Isolated Perfused Rabbit Liver, 136

BENNETT, LESLIE L., see HAZELWOOD, ROBERT L., 312

BENTLEY, P. J. The Effects of Neurohypophysial Extracts on the Tadpole of the Frog, *Heleioporus eyrei*, 609

BINKLEY, FRANCIS AND GERALD M. CHRISTENSEN. An Effect of Vasopressin on Renal Weight, 307

BLANSHARD, GERALD, ARTHUR I. COHEN, JULIAN ARABEHETY AND SEYMOUR J. GRAY. Gastric Secretory Activity in Mice and Rats with Transplanted Hormone Secreting Adrenocortical Tumors, 1032

BLUMENTHAL, HERMAN T., see PICKMAN, DONALD S., 790

BOGDANOVE, E. M. AND S. A. D'ANGELO. The Effects of Hypothalamic Lesions on Goitrogenesis and Pituitary TSH Secretion in the Propylthiouracil-treated Guinea Pig, 53

BORTLE, L., see PERRINE, J. W., 437

BOWERS, WILLIAM, see CLARK, IRWIN, 849

BOWMAN, BARBARA J., see GLENN, E. MYLES, 379

BOWMAN, BARBARA J., see GLENN, E. MYLES, 390

BOWMAN, B. J., see GLENN, E. MYLES, 419

BRADEN, A. W. H., see LAMOND, D. R., 921

BRENNAN, DAVID M. AND M. X. ZARROW. Water and Electrolyte Content of the Uterus of the Intact and Adrenalectomized Rat Treated with Relaxin and Various Steroid Hormones, 907

BROWN, JOSEPH D., see HOLLANDER, VINCENT P., 621

BURAK, ETHEL, AND J. M. WOLFE, The Effect of Anterior Hypophysea! Administration on the Ovaries of Old Rats, 676

CAÑADELL, JOSÉ, M., AND JOAQUIN BARRAQUER. Enhancing Effect of Corticotrophin on Thyrotrophin Produced Exophthalmos in Fishes, 1017

CARRIÈRE, RITA AND H. ISLER. Effect of Frequent Housing Changes and of Muscular Exercise on the Thyroid Gland of Mice, 414

CASENTINI, S., A. DE POLI, S. HUKOVIC AND L. MARTINI. Studies on the Control of Corticotrophin Release, 483

CLARK, IRWIN, RAYMOND F. GEOFFROY AND WILLIAM BOWERS. Effects of Adrenal Cortical Steroids on Calcium Metabolism, 849

CHAIKOFF, I. L., see TAUROG, ALVIN, 1038

CHENG, DOROTHY WEI. Effect of Progesterone and Estrone on the Incidence of Congenital Malformations Due to Maternal Vitamin E Deficiency, 270

CHRISTENSEN, GERALD M., see BINKLEY, FRANCIS, 307

CHRISTOPHE, JEAN AND JEAN MAYER. Effects of Acute and Chronic Treatments with Carbutamide (BZ-55) on Obese-Hyperglycemic Mice and Their Lean Littermates, 664

COHEN, ARTHUR I., see BLANSHARD, GERALD, 1032

CONTOPOULOS, ALEX N. AND MIRIAM E. SIMPSON. Growth-Promoting Activity of Pregnant Rat Plasma after Hypophysectomy and After Thyroidectomy, 1023

CRANE, W. A. J., R. E. PORTER AND D. J. INGLE. Pathologic Changes in Adrenalectomized Sensitized Rats Treated with Adrenal Cortex Extract, 215

D'ANGELO, S. A., see BOGDANOVE, E. M., 53

D'ANGELO, S. A. Thyroid Hormone Administration and Ovarian and Adrenal Activity in Rats Bearing Hypothalamic Lesions, 685

DAVIS, NEIL C., see MIRSKY, I. ARTHUR, 992

DENT, JAMES NORMAN. A Technique for Removing Thyroid Glands from Anuran Larvae, 314

DE POLI, S., see CASENTINI, S., 483

DEWIED, DAVID AND I. ARTHUR MIRSKY. The Action of Δ^1 Hydrocortisone on the Antidiuretic and Adrenocorticotropic Responses to Noxious Stimuli, 955

DIERMEIER, HAROLD F., see DI STEFANO, HENRY S., 448

DINERSTEIN, JENNY, see LANMAN, JONATHAN T., 494

DI STEFANO, HENRY S., AND HAROLD F. DIERMEIER. Effects of Restricted Food Intake and Growth Hormone on Rat Liver Proteins and Nucleic Acids, 448

DOCTOR, V. M. AND J. B. TRUNNELL. Studies on the Effect of Storage on the Purity of Sodium Iodide-I^{131}, 455

DORFMAN, RALPH I., see PERON, FERNAND G., 431

DORFMAN, RALPH I. The Anti-Androtenic Activity of a Phenanthrene Derivative in the Chick, 464

DOUGHMAN, D. J., see HALMI, N. S., 618

DRUET, DEA, see HART, KATHLEEN, T., 857

DURBIN, PATRICIA W., see ASLING, C. WILLET, 579

DYRENFURTH, INGE AND ELEANOR H. VENNING. Studies on the Assay of Aldosterone: Chromatography and Chemical Determination, 648

ECKSTEIN, B., see PERKE, M., 831

ELLIS, STANLEY, see HAZELWOOD, ROBERT L., 312

ENDAHL, GERALD L. AND CHARLES D. KOCHAKIAN. Influence of Androgens on Enzymes of the Krebs Cycle, 833

ENGEL, FRANK L. AND JOAN FREDERICKS. An Effect of Corticotropin on Urea Formation in Adrenalectomized-Nephrectomized Rats, 409

ENGEL, LEWIS L., see BAGGETT, BILLY, 600

ERB, R. E., see GORSKI, J., 707

ESHKOL, Z., see PEREK, M., 831

ETLING, NICOLE AND S. B. BARKER. Metabolism of Thyroxine During Prolonged Kidney Cortex Incubation, 753

EVANS, E. S., see SIMPSON, M. E., 592

EVERSOLE, W. J., see LAUBER, JEAN KAUTZ, 316

FARRELL, GORDON L., see ANDERSON, CHARLES H., 202

FELDMAN, DIANE, see MONEY, WILLIAM L., 123

FISKE, VIRGINIA M. AND ROY O. GREEP. Neurosecretory Activity in Rats Under Conditions of Continuous Light or Darkness, 175

FISHER, JAMES W. The Effects of Cobalt Injections on Total Circulating Red Cell Volume and Bone Marrow Cytol-

ogy in Normal and Adrenalectomized Dogs, 522

FORBES, THOMAS R. Rapid Disappearance of Progesterone from Oily Solutions Injected Intraperitoneally in Mice, 567

FORD, DONALD H., STRATOS KANTOUNIS AND ROBERT LAWRENCE. The Localization of I^{131}-Labelled Triiodothyronine in the Pituitary and Brain of Normal and Thyroidectomized Male Rats, 977

FORTIER, CLAUDE, see GUILLEMIN, ROGER, 310

FRANZ, JOHN M., see HORTON, H. ROBERT, 258

FREDERICKS, JOAN, see ENGEL, FRANK L., 409

FREINKEL, NORBERT AND SIDNEY H. INGBAR. The Effects of the Arylsulfonylureas on the Respiratory Activity and Glucose Metabolism of Isolated Rabbit Kidney Cortex, 1002

FREY, HARALD M. AND A. ALBERT. A Modification of the Bates-Cornfield Bio-Assay for Thyrotropin, 304

GAAL, STEPHEN A., see BATTS, ADRIENNE A., 503

GALE, C., see MCCANN, S. M., 870

GALTON, VALERIE A. AND ROSALIND PITT-RIVERS. The Effect of Excessive Iodine on the Thyroid of the Rat, 835

GEOFFROY, RAYMOND F., see CLARK, IRWIN, 849

GIROUD, CLAUDE J. P., see STACHENKO, JANINE, 730

GIROUD, CLAUDE J. P., see STACHENKO, JANINE, 743

GITELSON, SIMON, see MIRSKY, I. ARTHUR, 766

GLENN, E. MYLES. *In Vitro* and *in Vivo* Metabolism of Prednisolone: Studies Concerning Its Biological Effectiveness, 373

GLENN, E. MYLES, SARA LEE RICHARDSON AND BARBARA J. BOWMAN. A Method of Assay of Antitumor Activity using a Rat Mammary Fibroadenoma, 379

GLENN, E. MYLES, SARA LEE RICHARDSON, STANLEY C. LYSTER AND BARBARA J. BOWMAN. Inhibition of Mammary Fibroadenoma of Female Rats by Steroids of the Androstane Series, 390

GLENN, E. MYLES, S. C. LYSTER, B. J. BOWMAN AND S. L. RICHARDSON. Potentiation of Biological Activities of Steroids by Carcinogenic Hydrocarbons, 419

GORSKI, J. AND R. E. ERB. Characterization of Estrogens in the Bovine, 707

GRANNER, D. K., see HALMI, N. S., 618

GRANT, WILLIAM C., JR. A Test for Prolactin Using the Hypophysectomized Eft Phases of *Diemictylus Viridescens*, 839

GRAY, SEYMOUR J., see BLANSHARD, GERALD, 1032

GREENSPAN, FRANCIS S. AND WILLIAM LEW. Limitations of the Chick Radiophosphorus Uptake Assay and the Chick Radioiodine Depletion Assay for Thyrotropic Hormone When Applied to Blood and Urine, 160

GREEP, ROY O., see FISKE, VIRGINIA M., 175

GREER, MONTE A., see YAMADA, TAKASHI, 559

GREER, MONTE A. Correlations of Radioiodine Uptake with Thyroid Weight in the Mouse at Varying Levels of Exogenous and Endogenous Thyrotropin Stimulation, 724

GREER, DAVID S. The Distribution of Radioactivity in Non-Excretory Organs of the Male Rat after Injection of Testosterone-4-C^{14}, 898

GREIF, ROGER L. AND JEAN MORONEY. A Comparison of the Effects of *in Vivo* and *in Vitro* Thyroid Hormones upon the Oxygen Consumption of Rat Kidney Slices, 937

GUILLEMIN, ROGER, CLAUDE FORTIER AND HARRY S. LIPSCOMB. Comparison of *in Vitro* and *in Vivo* Assaying Procedures for Rat Adenohypophysial Corticotrophin, 310

GUILLEMIN, ROGER, see NICHOLS, BUFORD, JR., 914

HALBERG, FRANZ, RALPH E. PETERSON AND ROBERT H. SILBER. Phase Relations of 24-Hour Periodicities in Blood Corticosterone, Mitoses in Cortical Adrenal Parenchyma, and Total Body Activity, 222

HALMI, N. S. AND R. G. STUELKE. Comparison of Thyroidal and Gastric Iodide Pumps in Rats, 103

HALMI, N. S., H. ALBERT, D. J. DOUGHMAN, D. K. GRANNER AND B. N. SPIRTOS. Improved Intravenous Glu-

cose Tolerance in Thyroidectomized or Hypophysectomized Rats Treated with Triiodothyronine, 618

HART, KATHLEEN T., DEA DRUET AND ROBERT E. MACK. Quantitative *in Vitro* Response of Thyroid Tissue to Thyrotropic Hormone, 857

HAWKER, R. W., V. S. ROBERTS AND C. F. WALMSLEY. Oxytocin and Oxytocic Substance in Blood Extracts Before, and Following, Copulation in Sheep, 309

HAZELWOOD, ROBERT L., LESLIE L. BENNETT AND STANLEY ELLIS. Modification of the Catabolic Action of ACTH in Rats Fed a Diet High in Potassium, 312

HERBST, ARTHUR L., see URQUHART, JOHN F., 816

HEYDER, E., See PERRINE, J. W., 437

HISAW, FREDERICK L., JR. Comparative Effectiveness of Estrogens on Fluid Imbibition and Growth of the Rat's Uterus, 276

HOLLANDER, NINA, see HOLLANDER, VINCENT P., 621

HOLLANDER, VINCENT P., NINA HOLLANDER AND JOSEPH D. BROWN. The Activity of Synthetic Isoestrones in Pyridine Nucleotide Transhydrogenation, 621

HOLTHAUS, FRED J., JR., see LERNER, LEONARD J., 1010

HOLUB, DONALD A., see KITAY, JULIAN I., 475

HORTON H. ROBERT AND JOHN M. FRANZ. Effect of Ethionine on the Cortisone-Evoked Stimulation of Tryptophan Peroxidase-Oxidase Activity, 258

HUKOVIC, S., see CASENTINI, S., 483

INGBAR, S. H., B. A. ASKONAS AND T. S. WORK. Observations Concerning the Heterogeneity of Ovine Thyroglobulin, 110

INGBAR, SIDNEY H., see FREINKEL, NORBERT, 1002

INGLE, D. J., see CRANE, W. A. J., 215

INTOCCIA, ALFRED AND L. VAN MIDDLESWORTH. Thyroxine Excretion Increase by Cold Exposure, 462

ISLER, H., see CARRIÈRE, RITA, 414

ISLER, H. The Acute Effect of Salts on the Urinary Excretion of Radioiodide in Mice, 769

ISLER, H. The Effect of Adrenal and Pituitary Hormones on the Urinary Excretion of Iodide in Mice, 807

JAGIELLO, GEORGIANA, see VAN MIDDLESWORTH, LESTER, 186

JAILER, JOSEPH W., see KITAY, JULIAN I., 475

JENKINS, JOHN S., J. WILLIAM MEAKIN AND DON H. NELSON. A Comparison of the Inhibitory Effects of 2-methyl-1,2-bis(3-pyridyl)-1-propanone and Amphenone B on Adrenal Cortical Secretion in the Dog, 572

JOHNSTON, MURIEL E., see ASLING, C. WILLET, 579

KALANT, N., W. C. MCINTYRE AND D. L. WILANSKY. Thyroid Function in Experimental Nephrotic Syndrome, 333

KALLISTRATOS, G. AND K. D. VOIGT. The Influence of Cortisone Glycinate and Testosterone Glycinate on Adrenalectomized Golden Hamsters and Control Hamsters, 231

KALLMAN, BURTON AND PAUL STARR. The Effects of Epinephrine on Thyroxine Metabolism: Iodine Excretion after Various Thyronine Derivatives, 703

KAMBARA, SHUNICHI, see KOBAYASHI, HIDESHI, 615

KANTOUNIS, STRATOS, see FORD, DONALD H., 977

KENNY, ALEXANDER D. AND PAUL L. MUNSON. A Method for the Biological Assay of Phosphaturic Activity in Parathyroid Extracts, 513

KITAY, JULIAN I., DONALD A. HOLUB AND JOSEPH W. JAILER. Inhibition of Pituitary ACTH Release: An Extra-Adrenal Action of Exogenous ACTH, 475

KLITGAARD, HOWARD M., see KOT, PETER A., 319

KOBAYASHI, HIDESHI AND SHUNICHI KAMBARA. Alkaline Phosphatase Activity in the Pituitary Body of the Rat, 615

KOCHAKIAN, CHARLES D., see ENDAHL, GERALD L., 833

KONEFF, ALEXEI A., see VAN DYKE, DONALD C., 240

KOT, PETER A. AND HOWARD M. KLITGAARD. Elimination of C^{14}-Labeled Thyroxine in the Bile, Urine and Expired Air of Rats in Altered Thyroid States, 319

KROC, ROBERT L., see STASILLI, NEIL R., 62

LAMOND, D. R. AND A. W. H. BRADEN. Diurnal Variation in Response to Gonadotropin in the Mouse, 921

LANMAN, JONATHAN T. AND JENNY DINERSTEIN. The Adrenotropic Action of Human Pregnancy Plasma, 494

LANMAN, GERTRUDE, see BAGGETT, BILLY, 600

LARSON, FRANK C., see ALBRIGHT, EDWIN C., 208

LAUBER, JEAN KAUTZ AND W. J. EVERSOLE. The Use of the Toad for Bioassay of Mammalian Antidiuretic Hormone (Vasopressin), 316

LAWRENCE, ROBERT, See FORD, DONALD H., 977

LERNER, LEONARD J., FRED J. HOLTHAUS, JR. AND CHARLES R. THOMPSON. A Myotrophic Agent and Gonadotrophin Inhibitor, 19-Nortestosterone-17-benzoate, 1010

LEVY, ALAN C. AND ESTELLE R. RAMEY. The Effect of Adrenal Steroids on Peri-heral Fat Mobilization in the Rat, 586

LEW, WILLIAM, see GREENSPAN, FRANCIS S., 160

LIEBERMAN, AMOS H., see MULROW, PATRICK J., 631

LIPNER, H. J., BILLIE P. WAGNER AND HAROLD P. MORRIS. Depression of the Mouse Thyroid Iodide Pump by Goitrogens, 290

LIPSCOMB, HARRY S., see GUILLEMIN, ROGER, 310

LUETSCHER, JOHN A., JR., see MULROW, PATRICK J., 631

LYON, JOHN B., JR. Cardiac Glycogen and the Effect of Growth Hormone Among Inbred Strains of Mice, 967

LYSTER, STANLEY C., see GLENN, E. MYLES, 390

LYSTER, S. C., see GLENN, E. MYLES, 419

MACK, ROBERT E., see HART, KATHLEEN, T., 857

MACK, ROBERT, see MCCANN, S. M., 870

MARTINI, L., see CASENTINI, S., 483

MATOVINOVIC, JOSIP AND AUSTIN L. VICKERY. Relation of Nucleic Acids to the Structure and Function of the Guinea Pig Thyroid Gland, 149

MAYER, JEAN, see CHRISTOPHE, JEAN, 664

MCCALLY, MICHAEL, see ANDERSON, CHARLES H., 202

MCCANN, S. M., ROBERT MACK AND C. GALE. The Possible Role of Oxytocin in Stimulating the Release of Prolactin, 870

MCINTYRE, W. C., see KALANT, N., 333

MEAKIN, J. WILLIAM, see JENKINS, JOHN S., 572

MELTZER, ROBERT I., see STASILLI, NEIL R., 62

MELTZER, ROBERT I., see MONEY, WILLIAM L., 123

MEYER, ROLAND K., M. APPASWAMY RAO AND RICHARD L. ASPINALL. Inhibition of the Development of the Bursa of Fabricius in the Embryos of the Common Fowl by 19-nortestosterone, 890

MIRSKY, I. ARTHUR, SIMON GITELSON AND GLADYS PERISUTTI. The Effect of Tolbutamide on the Diabetogenic Action of Somatotropin, 766

MIRSKY, I. ARTHUR, see DE WIED, DAVID, 955

MIRSKY, I. ARTHUR, GLADYS PERISUTTI AND NEIL C. DAVIS. The Destruction of Glucagon by the Blood Plasma from Various Species, 992

MOGGIAN, GIUSEPPE. Progestational Activity of 17-α-Methyl-19-Nor-Testosterone, 363

MONEY, WILLIAM L., ROBERT I. MELTZER, DIANE FELDMAN AND RULON W. RAWSON. The Effect of Various Thyroxine Analogues on Suppression of I^{131} Uptake by the Rat Thyroid, 123

MORONEY, JEAN, see GREIF, ROGER L., 937

MORRIS, HAROLD P., see LIPNER, H. J., 290

MOUDGAL, N. R., E. RAGHUPATHY AND P. S. SARMA. Effect of Thyroid Imbalance on the Detoxication of Benzoic Acid in the Rat, 326

MULROW, PATRICK J., GEORGE L. SHMAGRANOFF, AMOS H. LIEBERMAN, CHARLES I. SLADE AND JOHN A. LUETSCHER, JR. Stimulation of Rat Adrenocortical Secretion by a Factor Present in Human Urine, 631

MUNSON, PAUL L., see KENNEY, ALEXANDER D., 513

MURRELL, L. R. AND PAUL FOLEY NACE. Experimental Diabetes in the Catfish: Normal and Alloxan-Diabetic Blood Glucose and Pancreatic Histology, 542

NACE, PAUL FOLEY, see MURRELL, L. R., 542

NELSON, DON H., see JENKINS, JOHN S., 572

NICHOLS, BUFORD, JR. AND ROGER GUILLEMIN. Endogenous and Exogenous Vasopressin on ACTH Release, 914
NOWELL, N. W. Studies in the Activation and Inhibition of Adrenocorticotrophin Secretion, 191

ORGEL, MICHAEL, see PICKMAN, DONALD S., 790

PARTRIDGE, R., see PERRINE, J. W., 437
PARROTT, MARSHALL W., see ASLING, C. WILLET, 579
PEREK, M., B. ECKSTEIN AND Z. ESHKOL. The Effect of ACTH on Adrenal Ascorbic Acid in Laying Hens, 831
PERISUTTI, GLADYS, see MIRSKY, I. ARTHUR, 766
PERISUTTI, GLADYS, see MIRSKY, I. ARTHUR, 922
PÉRON, FERNAND G. AND RALPH I. DORFMAN. A Method for the Evaluation of Adrenocortocotropic Hormone Suppressing Action of Corticoids, 431
PERRINE, J. W., L. BORTLE, E. HEYDER, R. PARTRIDGE, E. K. ROSS AND I. RINGLER. Adrenal Corticoid Activities of 9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-Pregnadiene-3,20-Dione, 437
PETERSON, RALPH E., see HALBERG, FRANZ, 222
PICKMAN, DONALD S., ALICE RIDLEY, MICHAEL ORGEL AND HERMAN T. BLUMENTHAL. Effect of Cortisone on Growth of Chick Embryos During Early Embryogenesis, 790
PITTMAN, C. S. AND S. B. BARKER. Inhibition of Thyroxine Action by 3,3′,5′-Triiodothyronine, 466
PITT-RIVERS, ROSALIND, see GALTON VALERIE A., 835
PITT-RIVERS, ROSALIND AND J. WOLFF. Chromatographic Artifacts Obtained with Sodium Iodide-I^{131}, 841
PONSETI, IGNACIO V. Studies on the Suppression of Aminonitrile Lesions in Rats by Thyroxine Analogues, 795
PORTER, JOHN C., see RUMSFELD, H. W., JR., 942
PORTER, JOHN C. AND H. W. RUMSFELD, JR. Further Study of an ACTH-Releasing Protein from Hypophyseal Portal Vessel Plasma, 948
PORTER, R. E., see CRANE, W. A. J., 215
POTTER, G. D., see TAUROG, ALVIN, 1038
PRUDDEN, JOHN F., see BECKER, DAVID V., 136

RAGHUPATHY, E., see MOUDGAL, N. R., 326
RALL, J. E., see WOLFF, J., 1
RALL, J. E., see ROBBINS, JACOB, 12
RALL, J. E., see ROBBINS, JACOB, 37
RAMEY, ESTELLE R., see LEVY, ALAN C., 586
RANNEY, R. E. The Effect of Estrogens on Bone Phosphate Accretion Resorption, and Exchange in Mice, 783
RASMUSSEN, HOWARD. Effect of the Injection Medium upon the Biological Response to Parathyroid Hormones, 367
RAWSON, RULON W., see MONEY, WILLIAM L., 123
RICHARDSON, SARA LEE, see GLENN, E. MYLES, 379
RICHARDSON, SARA LEE, see GLENN, E. MYLES, 390
RICHARDSON, S. L., see GLENN, E. MYLES, 419
RIDLEY, ALICE, see PICKMAN, DONALD S., 790
RINGLER, I., see PERRINE, J. W., 437
ROBBINS, JACOB, see WOLFF, J., 1
ROBBINS, JACOB, J. WOLFF AND J. E. RALL. Iodoproteins in Thyroid Tissue and Blood of Rats with a Transplantable Thyroid Tumor, 12
ROBBINS, JACOB, J. WOLFF AND J. E. RALL. Iodoproteins in Normal and Abnormal Human Thyroid Tissue and in Normal Sheep Thyroid, 37
ROBERTS, V. S., see HAWKER, R. W., 309
ROSE, GEORGE G. AND JACK B. TRUNNELL. Thyroid Epithelium in Tissue Cultures: Observations on the Morphology and Functional Capacities of Embryo Chick Thyroids, 344
ROSENBERG, I. N. Behavior of Iodide in Acid Solutions of Hemoglobin, 83
ROSENBERG, L. L., see SIMPSON, M. E., 592
ROSENKRANTZ, HARRIS. A Direct Influence of 5-Hydroxytryptamine on the Adrenal Cortex, 355
ROSS, E. K., see PERRINE, J. W., 437
RUMSFELD, H. W., JR. AND JOHN C. PORTER. Investigation of the Release of ACTH, 942
RUMSFELD, H. W., JR., see PORTER, JOHN C., 948

SANDBERG, AVERY A., NATHAN BACK, J. R. SCHUBARG AND W. ROY SLAUNWHITE, JR. Distribution of C^{14}-Estrone in Rats and Dogs Before and After Plasmapheresis, 775

SANTISTEBAN, GEORGE A. Adrenocortical Influences upon the Reconstitution of Lymphatic Tissue Following Acute Involution, 638
SARMA, P. S., see MOUDGAL, N. R., 326
SAYERS, GEORGE, see SOLOMON, NEIL, 535
SCHUBARG, J. R., see SANDBERG, AVERY A., 775
SHMAGRANOFF, AMOS H., see MULROW, PATRICK J., 631
SILBER, ROBERT H., see HALBERG, FRANZ, 222
SIMPSON, MIRIAM E., see VAN DYKE, DONALD C., 240
SIMPSON, M. E., E. S. EVANS AND L. L. ROSENBERG. Re-evaluation of the Evidence for a Pituitary Erythropoietic Hormone, 592
SIMPSON, MIRIAM E., see CONTOPOULOS, ALEX N., 1023
SLADE, CHARLES I., see MULROW, PATRICK J., 631
SLAUNWHITE, W. ROY, JR., see SANDBERG, AVERY A., 775
SLINGERLAND, D. W., see YAMAZAKI, E., 126
SOLOMON, NEIL, RANDALL H. TRAVIS AND GEORGE SAYERS. Corticosteroids and the Functional Capacity of the Rat Heart-Lung Preparation, 535
SPAZIANI, EUGENE AND CLARA M. SZEGO, 713. Further Evidence for Mediation by Histamine of Estrogenic Stimulation of the Rat Uterus, 713
SPIRTOS, B. N., see HALMI, N. S., 618
STACHENKO, JANINE AND CLAUDE J. P. GIROUD. Functional Zonation of the Adrenal Cortex: Pathways of Corticosteroid Biogenesis, 730
STACHENKO, JANINE AND CLAUDE J. P. GIROUD. Functional Zonation of the Adrenal Cortex: Site of ACTH Action, 743
STARR, PAUL, see KALLMAN, BURTON, 703
STASILLI, NEIL R., ROBERT L. KROC AND ROBERT I. MELTZER. Antigoitrogenic and Calorigenic Activities of Thyroxine Analogues in Rats, 62
STUELKE, R. G., see HALMI, N. S., 103
STURTEVANT, F. M. Prevention of Adrenal-Regeneration Hypertension by an Aldosterone-Blocker, 299
SZEGO, CLARA M., see SPAZIANI, EUGENE, 713

TAUROG, ALVIN, G. D. POTTER AND I. L. CHAIKOFF. The Effect of Hypophysectomy and of TSH on the Mouse Submaxillary Iodide Pump. 1038
THOMPSON, CHARLES R., see LERNER, LEONARD J., 1010
TIPTON, JR., DALE L., see BATTS, ADRIENNE A., 503
TOBIAS, CORNELIUS A., see VAN DYKE, DONALD C., 240
TOMITA, KENKICHI, see ALBRIGHT, EDWIN C., 208
TRAVIS, RANDALL H., see SOLOMON, NEIL, 535
TROOP, ROBERT C. Influence of Gonadal Hormones on the Metabolism of Cortisone, 671
TRUNNELL, JACK B., see ROSE, GEORGE G., 344
TRUNNELL, J. B., see DOCTOR, V. M., 455

URQUHART, JOHN F., EUGENE YATES AND ARTHUR L. HERBST. Hepatic Regulation of Adrenal Cortical Function, 816

VANDERLAAN, W. P., see VAN MIDDLESWORTH, LESTER, 186
VAN DYKE, DONALD C., MIRIAM E. SIMPSON, ALEXEI A. KONEFF AND CORNELIUS A. TOBIAS. Long Term Effects of Deuteron Irradiation of the Rat Pituitary, 240
VAN MIDDLESWORTH, LESTER, GEORGIANA JAGIELLO AND W. P. VANDERLAAN. Observations on the Production of Goiter in Rats with Propylthiouracil and on Goiter Prevention, 186
VAN MIDDLESWORTH, L., see INTOCCIA, ALFRED, 462
VENNING, ELEANOR H., see DYRENFURTH, INGE, 648
VICKERY, AUSTIN L., see MATOVINOVIC, JOSIP, 149
VOIGT, K. D., see KALLISTRATOS, G., 231

WAGNER, BILLIE P., see LIPNER, H. J., 290
WALMSLEY, C. F., see HAWKER, R. W., 309
WEXLER, BERNARD C. Lipid Depletion of the Zona Glomerulosa of the Rat Adrenal Cortex after Administration of 9-Alpha-Fluorohydrocortisone, 300
WILANSKY, D. L., see KALANT, N., 333
WILLIAMS, DAVID D. Limb Regeneration in the Salamander, *Triturus veridescens*, After Large Initial and During Prolonged Smaller Injections of Cortisone Acetate, 292
WOLFE, J. M., see BURACK, ETHEL, 676
WOLFF, J., JACOB ROBBINS AND J. E.

RALL. Iodide Trapping without Organification in a Transplantable Rat Thyroid Tumor, 1
WOLFF, J., see ROBBINS, JACOB, 12
WOLFF, J., see ROBBINS, JACOB, 37
WOLFF, J., see PITT-RIVERS, ROSALIND, 841
WORK, T. S., see INGBAR, S. H., 110
WRIGHT, PAUL A. Blood Sugar Studies in the Bullfrog, *Rana Catesbiana*, 551

YAMADA, TAKASHI AND MONTE A. GREER. Studies on the Mechanism of Hypothalamic Control of Thyrotropin Secretion: Effect of Thyroxine Injection into the Hypothalamus or the Pituitary on Thyroid Hormone Release, 559
YAMAZAKI, E. AND D. W. SLINGERLAND. The *in vitro* Metabolism of Thyroxine, Triiodothyronine and Their Acetic and Propionic Acid Analogues, 126
YATES, F. EUGENE, see URQUHART, JOHN F., 816

ZARROW, M. X., see BRENNAN, DAVID M., 907

SUBJECT INDEX VOLUME 64

ACTH
activation and inhibition of ACTH secretion, 191
comparison of *in vitro* and *in vivo* assay procedures, 310
catabolic action of, with high potassium diet, 312
effect of, on urea formation in adrenalectomized-nephrectomized rats, 409
suppressing action of corticoids, method of evaluation, 431
inhibition of pituitary ACTH release by by exogenous ACTH, 475
studies on control of ACTH release, 483
adrenotropic action of human pregnancy plasma, 494
stimulation of adrenocortical secretion by human urine factor, 631
effect of, on adrenal ascorbic acid in laying hens, 831
endogenous and exogenous vasopressin and ACTH release, 914
investigation of release of, 942
-releasing protein in hypophyseal portal plasma, 948
Adrenal cortex
aldosterone secretion, effect of atrial stretch, 202
extract and pathologic changes, 215
mitoses and 24-hour periodicity, 222
cortisone glycinate and testosterone glycinate, effects on adrenalectomized and normal hamsters, 231
cortisone stimulating tryptophan peroxidase-oxidase activity, effect of ethionine, 258
cortisone acetate and limb regeneration in the salamander, 292
adrenal-regeneration hypertension, prevention by an aldosterone-blocker, 299
lipid depletion of the zona glomerulosa by 9-α-alpha-fluorohydrocortisone, 300
direct influence of 5-hydroxytryptamine, 355
prednisolone, *in vitro* and *in vivo* metabolism, 373
corticoid suppression of ACTH, 431
corticoid activities of 9α-fluoro-11 β, 16α, 17α, 21-tetrahydroxy-1,4-pregnadiene-3, 2-dione, 437
corticoids and function of heart-lung preparation, 535
inhibition of adrenal cortical secretion by amphenone B and 2-methyl-1, 2-bis (3-pyridyl)-1-propanone, 572
adrenal steroids and fat mobilization, 586
functional zonation of adrenal cortex, pathways of corticosteroid biogenesis, 730
site of ACTH action, 743
adrenal cortical function, hepatic regulation of, 816
adrenal steroids and calcium metabolism, 849
action of Δ^1 hydrocortisone on responses to noxious stimuli, 955
hormone secreting adrenal cortical tumors, effect on gastric secretory activity, 1032
Amphenone
inhibition of adrenal cortical secretion by amphenone B, 572
Androgens
progestational activity of 17α-methyl-19-nortestosterone, 363
anti-androgenic activity of phenanthrene derivative, 464
conversion of C^{14}-testosterone to C^{14}-estrogenic steroids, 600
influence of gonadal hormone on cortisone metabolism, 671
influence of, on Kreb's cycle enzymes, 833
inhibition of bursa of fabricius in chick embryos by 19-nortestosterone, 890
distribution of radioactivity in male non-excretory organs after injection of testosterone-4-C^{14}, 898
gonadotrophin inhibitor, 19-nortestosterone-17-benzoate, 1010

Blood sugar
studies of, in the bullfrog, 551

Estrogens
estrone and congenital malformations due to Vitamin E deficiency, 270
comparative effectiveness on uterine growth and water imbibition, 276
C^{14}-estrogenic steroids, conversion from C^{14}-testosterone by endocrine tissues, 600
activity of isoestrones in pyridine nucleotide transhydrogenation, 621
adrenal cortical hormones and reconstitution of lymphatic tissues, 638
influence of gonadal hormones on cortisone metabolism, 671
characterization of estrogens in the bovine, 707
estrogenic stimulation of the uterus, mediation by histamine, 713
distribution of C^{14}-estrone before and after plasmapheresis, 775
effect of estrogens on bone phosphate metabolism, 783

Glucagon
destruction by blood plasma from various species, 992

Gonadotropins
diurnal variation in response to gonadotropin in the mouse, 921
gonadotropin inhibition by 19-nortestosterone-17-benzoate, 1010

Growth hormone
effect of, and of restricted food intake on rat liver proteins and nucleic acids, 448
and hypophysectomy, effect on Golgi apparatus in islets of Langerhans, 610
effect of tolbutamide on the diabetogenic action of, 766
cardiac glycogen and, 967
growth-promoting activity of pregnant rat plasma after hypophysectomy and thyroidectomy, 1023

Hypophysis
long term effects of deutron irradiation, 240
erythropoietic hormone, re-evaluation of evidence, 592
alkaline phosphatase activity in the, 615

Hypothalamus
hypothalamic lesions and TSH secretion in the guinea pig, 53
thyroxine injection into, and TSH secretion, 559
hypothalamic lesions, thyroid hormone, and ovarian and adrenal activity, 685

Insulin
insulin resistance and inactivation in mice, 400
changes in Golgi apparatus in islets of Langerhans following glucose and insulin administration, 503

Mammary gland
assay of antitumor activity using rat mammary fibroadenoma, 379
inhibition of mammary fibroadenoma by androstane steroids, 390
astatine-211 in mammary tissue, 579

Muscle
effect of hormones on muscle atrophy regression, 262
myotrophic agent, 19-nortestosterone-17-benzoate, 1010

Neurohypophysis
effect of vasopressin on renal weight, 307
oxytocin in blood before and after copulation in sheep, 309
use of toad in bioassay of vasopressin, 318
effect of neurohypophysial extracts on tadpole, 609
possible role of oxytocin in prolactin release, 870

Ovary
effects of anterior hypophysial administration on ovaries of old rats, 676

Neurosecretion
continuous light or darkness and neurosecretory activity, 175

Pancreas
changes in the Golgi apparatus in islets of Langerhans following glucose and insulin administration, 503
normal and diabetic blood glucose and pancreatic histology in the catfish, 542
effect of hypophysectomy and growth hormone on Golgi apparatus of islets of Langerhans, 610

Parathyroids
influence of gelatin in the assay of purified extracts, 296
effect of injection medium on biological response to parathyroid hormone, 367
assay of phosphaturic activity in extracts, 513

Progesterone
progesterone and congenital malformations due to vitamin E deficiency, 270

progestational activity of 17α-methyl-19-nortestosterone, 363
rapid disappearance of, from oily solutions, 567
Prolactin
assay using the hypophysectomized eft, 839

Relaxin
water and electrolyte content of uterus of intact and adrenalectomized rats, 907

Steroids
potentiation of biological activities of, by carcinogenic hydrocarbons, 419

Thyroid
iodide trapping in tumor, 1
iodoproteins in thyroid tumor, 12
iodoproteins in human and sheep thyroid, 37
activities of thyroxine analogues in the rat, 62
behavior of iodide in acid solutions of hemoglobin, 83
thyroidal and gastric iodide pumps in rats, 103
heterogeneity of ovine thyroglobulin, 110
thyroxine analogues and suppression of I^{131} uptake by rat thyroid, 123
in vitro metabolism of thyroxine, triiodothyronine and their acetic and propionic acid analogues, 126
metabolism of thyroxine, triiodothyronine and diiodotyrosine by liver, 136
nucleic acids in guinea pig thyroid, 149
goiter production and goiter prevention in the rat, 186
in vitro metabolism of triiodothyronine, 208
thyroid iodide pump, inhibition by goitrogens, 290
thyroidectomy of Anuran larvae, 314
elimination of C^{14}-thyroxine, 319
thyroid imbalance and detoxication of benzoic acid, 326
thyroid function in nephrotic syndrome, 333
tissue culture of chick embryo thyroids, 344
housing changes and exercise, effect on thyroid, 414
effect of storage on purity of I^{131}-iodide, 455
thyroxine excretion, increased by cold exposure, 462
thyroid action, inhibition of 3, 3′,5′-triiodothyronine, 466
triiodothyronine and glucose tolerance in thyroidectomized and hypophysectomized rats, 618
thyroxine metabolism, effects of epinephrine, 703
thyroxine metabolism by kidney cortex, 753
excretion of radioiodide, effect of salts, 769
thyroxine analogues, suppression of aminonitrile lesions, 795
excretion of iodide, effect of adrenal and pituitary hormones, 807
effect of excessive iodine on rat thyroid, 835
iodide-I^{131}, chromatographic artifacts, 841
thyroid hormones, effects on oxygen consumption of kidney slices, 937
localization of triiodothyronine, 977
Thyrotropin
assay in blood and urine, 160
assay, modification of Bates-Cornfield method, 304
stimulation, effect on radioiodine uptake and thyroid weight, 724
in vitro response of thyroid tissue to TSH, 857
enhancing effect of ACTH on thyrotropin-induced exophthalmos in fishes, 1017
hypophysectomy and, effect on mouse submaxillary iodide pump, 1056

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Volume 64 JUNE, 1959 Number 6

ENDOCRINOLOGY

THE UNIVERSITY OF MICHIGAN
JUN 14 1959
MEDICAL

CLARK, IRWIN, RAYMOND F. GEOFFROY AND WILLIAM BOWERS
Effects of Adrenal Cortical Steroids on Calcium Metabolism 849

HART, KATHLEEN T., DEA DRUET AND ROBERT E. MACK
Quantitative *in vitro* Response of Thyroid Tissue to Thyrotropic Hormone ... 857

McCANN, S. M., ROBERT MACK AND C. GALE
The Possible Role of Oxytocin in Stimulating the Release of Prolactin 870

MEYER, ROLAND K., M. APPASWAMY RAO AND RICHARD L. ASPINALL
Inhibition of the Development of the Bursa of Fabricius in the Embryos of the Common Fowl by 19-nortestosterone ... 890

GREER, DAVID S.
The Distribution of Radioactivity in Non-Excretory Organs of the Male Rat after Injection of Testosterone-4-C^{14} 898

BRENNAN, DAVID M. AND M. X. ZARROW
Water and Electrolyte Content of the Uterus of the Intact and Adrenalectomized Rat Treated with Relaxin and Various Steroid Hormones 907

NICHOLS, BUFORD, JR. AND ROGER GUILLEMIN
Endogenous and Exogenous Vasopressin on ACTH Release 914

LAMOND, D. R. AND A. W. H. BRADEN
Diurnal Variation in Response to Gonadotropin in the Mouse 921

GREIF, ROGER L. AND JEAN MORONEY
A Comparison of the Effects of *in vivo* and *in vitro* Thyroid Hormones Upon the Oxygen Consumption of Rat Kidney Slices 937

RUMSFELD, H. W., JR. AND JOHN C. PORTER
Investigation of the Release of ACTH .. 942

PORTER, JOHN C. AND H. W. RUMSFELD, JR.
Further Study of an ACTH-Releasing Protein from Hypophyseal Portal Vessel Plasma 948

DE WIED, DAVID AND I. ARTHUR MIRSKY
The Action of Δ^1 Hydrocortisone on the Antidiuretic and Adrenocorticotropic Responses to Noxious Stimuli 955

LYON, JOHN B., JR.
Cardiac Glycogen and the Effect of Growth Hormone Among Inbred Strains of Mice 967

FORD, DONALD H., STRATOS KANTOUNIS AND ROBERT LAWRENCE
The Localization of I^{131}-Labeled Triiodothyronine in the Pituitary and Brain of Normal and Thyroidectomized Male Rats 977

MIRSKY, I. ARTHUR, GLADYS PERISUTTI AND NEIL C. DAVIS
The Destruction of Glucagon by the Blood Plasma from Various Species 992

FREINKEL, NORBERT AND SIDNEY H. INGBAR
The Effects of the Arylsulfonylureas on the Respiratory Activity and Glucose Metabolism of Isolated Rabbit Kidney Cortex 1002

LERNER, LEONARD J., FRED J. HOLTHAUS, JR. AND CHARLES R. THOMPSON
A Myotrophic Agent and Gonadotrophin Inhibitor, 19-Nortestosterone-17-benzoate 1010

CAÑADELL, JOSÉ M. AND JOAQUIN BARRAQUER
Enhancing Effect of Corticotrophin on Thyrotrophin Produced Exophthalmos in Fish 1017

CONTOPOULOS, ALEX N. AND MIRIAM E. SIMPSON
Growth-Promoting Activity of Pregnant Rat Plasma After Hypophysectomy and After Thyroidectomy 1023

BLANSHARD, GERALD, ARTHUR I. COHEN, JULIAN ARABEHETY AND SEYMOUR J. GRAY
Gastric Secretory Activity in Mice and Rats with Transplanted Hormone Secreting Adrenocortical Tumors 1032

TAUROG, ALVIN, G. D. POTTER AND I. L. CHAIKOFF
The Effect of Hypophysectomy and of TSH on the Mouse Submaxillary Iodide Pump 1038

FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY 1052

SOCIETY FOR ENDOCRINOLOGY 1053

INTER-SOCIETY CYTOLOGY COUNCIL 1053

INDEX 1054

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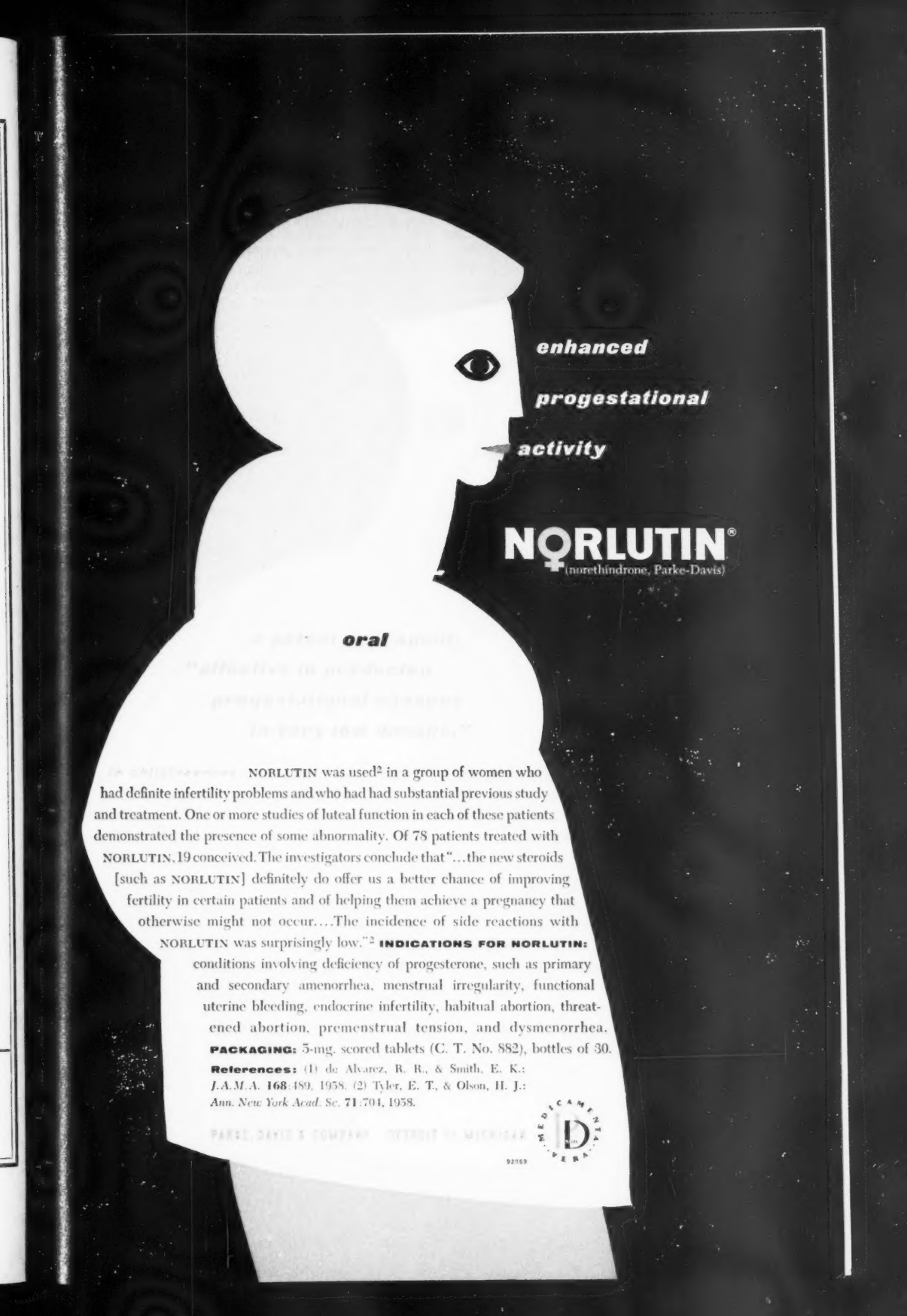

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